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Regulation of the Renin-Angiotensin-Aldosterone System by Reactive Oxygen Species

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Abstract

Angiotensin II (Ang II), the major effector of the renin-angiotensin-aldosterone system (RAAS), stimulates the production of reactive oxygen species (ROS) which are critically involved in Ang II-induced effects. Noteworthy, accumulating evidence indicates that ROS also regulate the activation of RAAS, contributing to the fine-tuning of this system under physiological conditions or to the amplification of the deleterious signaling in several pathologies. This chapter aims at giving an overview of the role of ROS in the regulation of expression, secretion and/or activity of several RAAS components.

Keywords: reactive oxygen species, superoxide, hydrogen peroxide, angiotensinogen, renin, pro(renin) receptor, angiotensin converting enzyme, angiotensin converting enzyme-2, angiotensin II, angiotensin 1–7, aldosterone, angiotensin II type 1 (AT_1) receptor, angiotensin II type 2 (AT_2) receptor, MAS receptor, regulation of expression, secretion or activity

1. Introduction

In the last two decades, reactive oxygen species (ROS) have emerged as downstream mediators of angiotensin II (Ang II) effects. The Ang II-induced activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases within the cardiovascular system, the kidney and the brain result in increased generation of ROS, such as superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), which are involved in diverse signaling functions. Interestingly, increasing evidence suggests that ROS also act as upstream regulators of the renin-angiotensin-aldosterone system (RAAS) in various cells and tissues. In several pathological conditions, ROS have been shown to increase RAAS activation, thus creating a vicious cycle that amplifies the deleterious signaling pathways orchestrated by this endocrine system. This chapter aims at

giving an overview of the interactions between ROS and RAAS, focusing on the effects of ROS on the expression, secretion and/or activity of RAAS components that may contribute to the development and progression of cardiometabolic and renal diseases.

2. ROS as regulators of the RAAS

2.1. ROS and angiotensinogen (AGT)

AGT is a 60-kDa α_2-globulin glycoprotein with 452 amino acids in humans (453 in rodents) that is mainly secreted by hepatocytes and constitutes the precursor of the RAAS [1]. AGT is a specific human substrate for renin which catalyzes the conversion of intact AGT into angiotensin I (Ang I), by releasing this decapeptide from the 63-residue NH_2-terminus.

The exact dynamics of AGT cleavage by renin has been a question of debate. In 2010, Zhou et al. suggested that the renin-cleavage site is normally in a buried position and that access and binding of renin to intact AGT would imply a conformational change that results from a disulfide bridge between two cysteines residues (Cys 18-138 in humans, Cys 18-137 in mouse) [2]—the only two conserved in all species [3, 4]. This disulfide bridge seems to be quite labile and both reduced and oxidized forms of AGT circulate in human plasma with a consistent reduced-to-oxidized ratio of 40:60 [2]. Furthermore, the study of Zhou et al. showed (although with no statistical analysis) that the affinity for renin is higher for the oxidized form of AGT when compared with the reduced form and that the affinity was even further increased in the presence of the (pro)renin receptor (PRR) [2]. These results suggest that prooxidant conditions might favor the oxidized conformation of AGT and, subsequently, activation of the RAAS. However, a very recent study of Wu et al. [5] challenged these data. They used AGT floxed mice which are almost depleted of liver-derived plasma AGT and, through the use of viral vectors specifically targeting hepatocytes, injected either wild-type AGT or AGT containing Cys18Ser and Cys137Ser mutants that were unable to form the disulfide bridge. The study showed that in mice most of the AGT exists in the oxidized bridged form and intriguingly, it was not possible to distinguish its effects on plasma renin and Ang II concentrations, in renal Ang II concentration or Ang II-dependent effects (increase in systolic blood pressure and pro-atherosclerotic effect in low-density lipoprotein (LDL) receptor$^{-/-}$ mice) [5]. So, it seems that at least in mice, the disulfide bridge is not relevant for the cleavage of AGT by renin in both the plasma and the kidney. However, species differences certainly exist and might be worth studying in the near future. In this context, it has recently been published a suggested protocol in order to modify commercially available enzyme-linked immunosorbent assay (ELISA) kits so that accurate measurements of intact AGT, in both oxidized and reduced forms, can be performed [6]. This will enable researchers to expand their studies and push forward the state-of-the-art on this field.

The evidence that ROS regulate the expression of AGT is mostly characterized in the kidney. The original study was performed in 2002, by Hsieh et al., who suggested that the mechanism through which high glucose induces AGT expression in immortalized renal proximal tubule cells (IRPTCs) was ROS generation [7]. They found that cultured IRPTCs stimulated with

high-glucose medium increased the accumulation of AGT mRNA and its secretion into the culture medium. This effect was blocked by taurine (an antioxidant), tiron (an $O_2^{\bullet-}$ scavenger), MnTBAP (a manganese-dependent superoxide dismutase (SOD) mimetic) and catalase (a H_2O_2 neutralizing enzyme), thus suggesting the involvement of ROS, namely $O_2^{\bullet-}$ and H_2O_2. Indeed, the increase in AGT mRNA accumulation and secretion was also observed when IRPTCs were directly stimulated with H_2O_2 in high-glucose but not in normal-glucose conditions. The stimulatory effect of high glucose on AGT expression via ROS has been further confirmed to occur in IRPTCs by the same group [8] and suggested to occur through mitogen-activated protein kinase (MAPK) activation [7] and also protein kinase C (PKC) and hexosamine biosynthesis pathway signaling [8]. ROS also mediate the effect of TGFβ1 on AGT expression. Again in IRPTCs, it was observed that TGFβ1 induced the expression of AGT mRNA and that this effect was blocked by tiron and diphenylene iodonium (DPI, an NADPH oxidase inhibitor) pointing to a ROS-mediated effect [9]. Once more, MAPK signaling seemed to be involved since the effect was blocked by SB203580, an inhibitor of p38 MAPK [9].

The role for ROS in mediating AGT expression has also been studied through a different approach that is the use of transgenic mice overexpressing catalase, therefore reducing the levels of endogenous H_2O_2. Using this approach, it was observed that overexpression of catalase specifically in the renal proximal tubule cells (RPTCs) decreased the renal expression of AGT (evaluated by immunohistochemistry, Western Blot (WB) and polymerase chain reaction (PCR)) compared to that found in wild-type (WT) control mice. Although this was not confirmed in another study using the same approach [10], it suggests that the regulatory effect of H_2O_2 over AGT expression might be physiological, at least in the RPTCs of the mice kidney. Brezniceanu et al. expanded this view and reported that *ex vivo* exposure of RPTCs from WT mice to high glucose or to Ang II increased the generation of ROS or AGT (mRNA or protein) but this increase was not observed in cells from transgenic mice overexpressing catalase in their RPTCs [11], suggesting that ROS-mediated AGT expression might also occur in high-glucose conditions. In line with this, induction of diabetes in mice with streptozotocin (STZ, an experimental model of type I diabetes) increased the expression of AGT (mRNA and protein), plasminogen activator inhibitor-1 (a marker of ROS-inducible gene), p53 and Bax mRNA (proapoptotic markers) in RPTCs but these effects were absent when STZ-diabetes was induced in transgenic mice overexpressing catalase in their RPTCs [11]. Also, the negative impact of catalase on AGT expression was also observed when overexpression of catalase was induced in RPTCs of Akita mice (a spontaneous genetic model of type 1 diabetes), which per se showed increased AGT expression compared with WT controls [12]. This was further confirmed in another study in which overexpression of catalase markedly attenuated the increase in the urinary excretion of AGT and Ang II [10]. Even though, catalase overexpression attenuated but did not prevent the alterations seen in the diabetic kidney [11, 12]. It was suggested that endogenous H_2O_2 stimulates nuclear, but not cytoplasmatic, Nrf2 (Nuclear factor erythroid 2-related factor 2, a master regulator of redox balance in cellular cytoprotective responses) levels that, in turn, stimulate intrarenal AGT expression and RAAS activation, possibly contributing to hypertension and development of nephropathy in the Akita model of diabetes [10]. This was suggested to be a tissue-specific regulatory mechanism since *in vivo* treatment with oltipraz, an Nrf2 activator, stimulates the expression of Nrf2 and AGT in

RPTCs but not the expression of AGT mRNA in the liver [10]. Taken together, these results highlight H_2O_2 as a key element in the regulatory effect of ROS over AGT expression.

Regulation of AGT expression by ROS has also been studied in kidney structures other than the RPTCs. The Zucker diabetic fatty (ZDF) rat is an experimental model of type II diabetes that develops diabetes by 17 weeks of age with renal injury starting between 18 and 20 weeks of age and being associated with oxidative stress [13]. Ohashi et al. observed that in 18-week-old ZDF rats, the immunoreactivity against AGT was increased in the glomeruli compared with that of the lean rat and that the majority of glomerular AGT staining was found in mesangial cells, although it was also found in podocytes [13]. Moreover, in primary cultures of rat mesangial cells from ZDF rats, H_2O_2 increased the expression of AGT mRNA and protein via phosphorylation of extracellular signal-regulated kinase (ERK), Jun kinase (JNK) but not p38 MAPK and these effects were suppressed by catalase treatment [13]. Also, culturing the rat glomerular mesangial cell line HBZY-1 in high-glucose conditions increased AGT mRNA levels and increased Ang II concentration in the culture media through activation of NADPH oxidase, since the inhibitor DPI abolished these effects in high-glucose but not under normal-glucose conditions [14]. The ROS-associated stimulation of AGT expression seems to be crucial for the pathophysiology of renal damage, at least in the ZDF rat, since increased urinary excretion of 8-isoprostanes (a marker of oxidative stress) and increased kidney AGT levels precede the development of renal damage [15, 16]. More generally in the kidney, we have also previously reported that in Ang II-induced hypertension there is an associated increase in the renal medullary (not cortical) production of H_2O_2 which induces the translocation of nuclear factor kappa B (NF-κB) p50/p50 homodimer and, subsequently, increases the renal production of AGT [17]. This was shown by direct measurements of H_2O_2 production and by the urinary excretion of AGT on Ang II-hypertensive animals and corroborated by the results from PEG-catalase-treated Ang II-hypertensive rats. Interestingly, this study from our group [17] raised the possibility for H_2O_2 to be a key element in the fine-tuning processes of AGT regulation. Indeed, we have also observed that both in normotensive Wistar and spontaneously hypertensive rats (SHR), STZ-induced diabetes was associated with an increase in the medullary production and urinary excretion of H_2O_2 and an increased AGT urinary excretion but a decreased plasma AGT concentration [18]. Of note, Ang II-hypertensive rats had also decreased plasma AGT concentration on day 14 of Ang II infusion, while PEG-catalase-treated Ang II-infused rats exhibited a marked increase in plasma AGT concentration [17].

The highly reactive $O_2^{\bullet-}$ has also been implicated in the regulation of AGT expression by ROS in the kidney. Feeding Dahl salt-sensitive rats with a high-salt diet increased blood pressure, urinary excretion of thiobarbituric reactive substances (TBARS) and kidney AGT protein levels while decreased plasma AGT levels [19]. *In vivo* treatment of these rats with tempol (a SOD mimetic) totally prevented the increase in the urinary excretion of TBARS, attenuated the hypertension and although it did not affect the plasma levels of AGT, it prevented the increase in kidney AGT levels and, subsequently, decreased kidney Ang II levels [19]. On the other hand, *in vivo* treatment with hydralazine was associated with similar reduction of blood pressure and no change in plasma levels of AGT, but only partially attenuated the urinary excretion of TBARS, did not prevent the increase in kidney AGT levels and actually increased kidney Ang II levels [19]. So, attenuation of ROS, namely of $O_2^{\bullet-}$, more than controlling

hemodynamic-mediated renal injury, it attenuates the tissue-specific increase in renal RAAS activity seen in Dahl salt-sensitive rats on a high-salt diet [19]. In endothelial nitric oxide synthase (eNOS)$^{-/-}$ mice, a high-salt diet also elevates blood pressure and causes progressive renal injury associated with increased glomerular $O_2^{\bullet-}$ production and urinary AGT excretion and renal AGT expression (mRNA and protein) [20]. This was observed mostly in the glomeruli (endothelial and mesangial cells) although also in the renal tubules [20]. Interestingly, the increase in $O_2^{\bullet-}$ production was seen immediately since the beginning of the high-salt diet, while the increase in AGT production started only 3 days after the beginning of the high-salt diet [20]. Once more, tempol prevented these effects [20]. Besides, tempol prevented the increased expression of AGT, renin and angiotensin-converting enzyme (ACE) mRNA and increased the levels of systemic and renal ROS observed in SHR rats on a high-fat diet [21].

Although, as previously said, evidence for ROS-mediated regulation of AGT expression comes mostly from studies concerning the kidney, other tissues have recently started to be analyzed. For instance, in primary cultures of cardiac fibroblasts, H_2O_2 induced a fivefold increase in AGT mRNA expression [22] and this effect might be relevant for the development of cardiac fibrosis since it was associated with increased collagen expression [22]. Also, human placenta explants subjected to experimental hypoxia-reperfusion for 24 h or treatment with H_2O_2 under normoxia increased AGT protein expression without affecting the expression of the other RAAS components [23]. Surprisingly, in the adipose tissue, ROS seem to downregulate the expression of AGT. Indeed, during adipocyte hypertrophy, ROS production increased along with inflammatory markers such as monocyte chemoattractant protein 1 (MCP-1) and interleukin 6 but AGT mRNA and secretion into the culture medium was decreased [24]. This was observed in differentiated 3T3-L1 adipocytes and in primary adipocytes. Inversely, treatment with the antioxidant N-acetylcysteine (NAC) suppressed the ROS production, inhibited the increase of the MCP-1 expression of hypertrophied adipocytes and increased AGT mRNA level [24]. Similar results were obtained in the obese db/db mice. In fact, compared with their lean littermates, the obese db/db mice showed decreased AGT mRNA in epididymal adipose tissue, but increased systemic and local tumor necrosis factor α (TNF-α) and oxidative stress [24]. Again, treatment with NAC reduced oxidative stress, interleukin 6 and TNF-α, but increased the AGT mRNA level in the epididymal adipose tissue, while liver AGT mRNA levels were not altered [24]. In this study, Okada et al. raised the hypothesis that tissue-specific decrease of AGT in obese adipose tissue may serve as a defense against further exacerbation of adiposity [24]. In line with this, we just recently observed (Morato et al., unpublished observations) that in obese prepubertal children, the duration of obesity seems to trigger a systemic H_2O_2/AGT pathway (eventually originated from the adipose tissue) that might help to control plasma AGT levels and, subsequently, Ang II-mediated increase in renal AGT expression and, thus, renal RAAS activation. Moreover, this interplay seems to be implicated in renal tissue remodeling since urinary excretion of AGT was associated with the urinary excretion of profibrotic cytokines endothelin 1 (ET-1) and transforming growth factor β (TGF-β) [25]. So, further studies are needed to expand the knowledge concerning the regulation of AGT expression by ROS in different tissues and experimental models of disease so that the big picture can be taken.

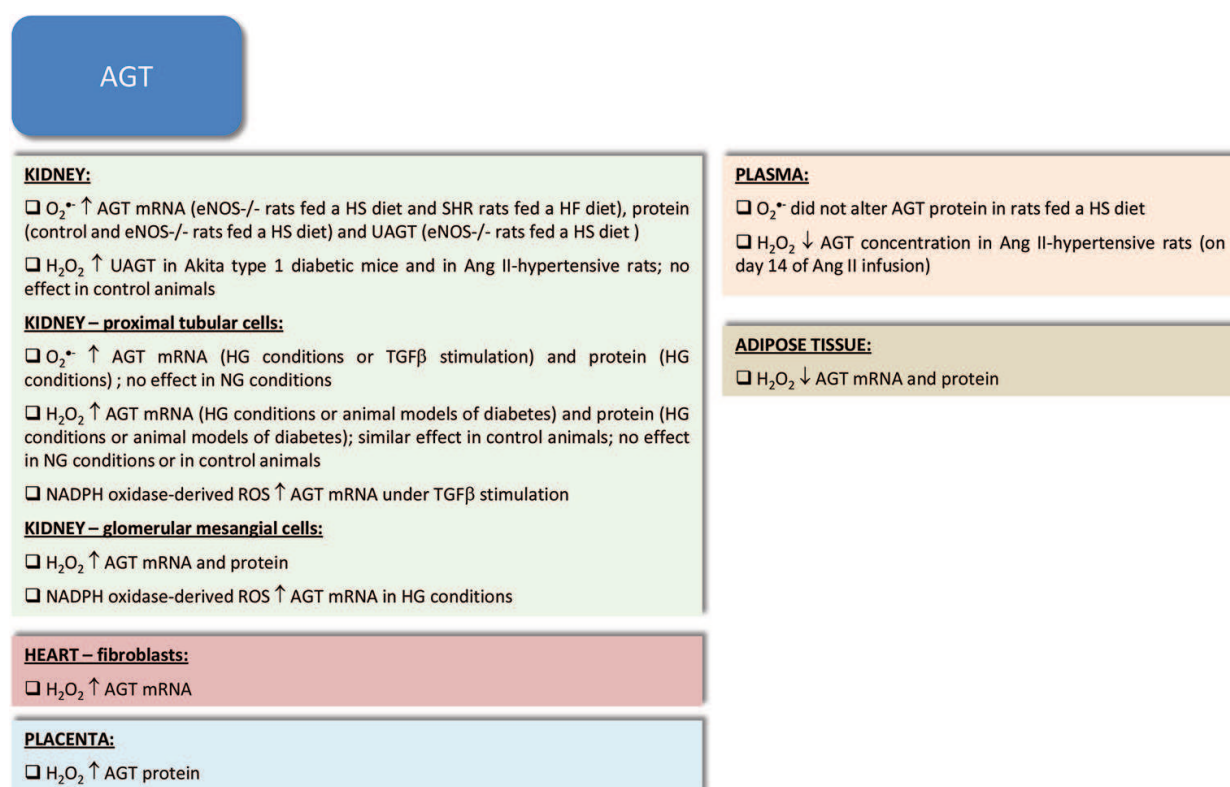**Figure 1** summarizes the role of ROS in the regulation of AGT.

Figure 1. Regulation of AGT by ROS. AGT, angiotensinogen; Ang II, angiotensin II; eNOS, endothelial nitric oxide synthase; HF, high-fat; HS, high-salt; NG, normal glucose; TGFβ, transforming growth factor beta; ROS, reactive oxygen species; SHR, spontaneously hypertensive rats; UAGT, urinary AGT.

2.2. ROS, renin and the prorenin receptor

Renin is the enzyme responsible for the initiation of the RAAS pathway. It is an aspartyl protease with high specificity toward AGT which is its only known substrate [26, 27]. Renin catalyzes the rate-limiting step of Ang II formation, cleaving 10 amino acids from the NH_2-terminus of AGT with resulting production of Ang I which is subsequently transformed into Ang II by ACE [28, 29]. Circulating active renin is predominantly derived from the juxtaglomerular (JG) cells in the renal afferent arterioles [26, 27, 30]. In the kidney, renin can also be synthesized, although to a lesser extent, in the renal proximal and connecting tubules and in the collecting duct [26, 31]. There are also extrarenal sources of renin where renin is generated as part of the tissue-specific RAAS, but in much lower levels than in the kidney [26].

Renin is initially produced as a preprorenin protein that is further cleaved originating prorenin. This renin precursor is either directed to dense-core secretory granules for controlled exocytosis or constitutively secreted [26, 32]. Directly released prorenin accounts for 80–90% of the total renin in human circulation [26, 30, 32]. Therefore, questions have arisen regarding the physiological role of prorenin, namely if circulating prorenin can be activated into renin, or if it acts independently of the formation of active renin, for example by binding to a specific receptor [26, 33]. This receptor has been identified and named PRR and can bind both prorenin and renin [26, 33, 34]. The catalytic activity of renin is fourfold increased when renin is bound to PRR [26]. The binding of prorenin to PRR also confers enzymatic activity to prorenin which then becomes able to convert AGT into Ang I, without proteolytic removal of the prosegment

[26, 33, 34]. Binding of prorenin and renin to PRR also triggers a range of intracellular events in the receptor-expressing cells, contributing to the upregulation of profibrotic genes [26, 33, 34]. Activation of renin occurs by proteolytic cleavage of prorenin within the secretory granules [26, 32]. It is currently not known if prorenin can be activated in the extracellular space, but it has been reported that it can be taken up by some tissues and contribute to the local production of angiotensin peptides [32, 35].

The initial evidence of the involvement of ROS in the regulation of renin came from the studies of Galle et al. [36–38]. The existence of ROS-producing cells in the close vicinity to JG cells led these authors to question if ROS modulate renin release [37]. In these studies, performed in primary cultured mouse JG cells, renin activity was measured by radioimmunoassay both in cells and supernatants and the renin release rates were expressed as the percentage of extracellular renin activity compared to the total renin activity [36–38]. The viability of cells after the incubation periods was tested and shown to be preserved [36–38]. It was found that the prolonged exposure (20 h) of JG cells to the $O_2^{\bullet-}$-generating xanthine/xanthine oxidase (XOD) reaction had a stimulatory effect on renin release. This increase was only modestly inhibited by the $O_2^{\bullet-}$-removing enzyme, SOD, but was eliminated by catalase, an H_2O_2-neutralizing enzymatic defense [37]. Furthermore, H_2O_2 applied exogenously for 20 h dose-dependently stimulated renin release and this effect was also prevented by catalase. Therefore, it was concluded that H_2O_2 or a subsequently formed reaction product, such as the hydroxyl radical ($^{\bullet}OH$), promotes renin release [37]. In subsequent studies, these authors investigated the effects of the treatment for 20 h with native and oxidized LDL and lipoprotein A (LpA) on renin release in JG cells, as well as the contribution of ROS to the putative lipoprotein-stimulated renin release [36, 38]. They observed that although renin release was not affected by native LDL or LpA, it was markedly stimulated by oxidized LDL and LpA, with oxidized LpA being about 30-fold more potent than oxidized LDL [36, 38]. SOD further enhanced the oxidized LpA-stimulated renin release but partly inhibited the renin release induced by oxidized LDL [38]. Catalase abolished the stimulatory effect of oxidized LpA on renin release, both in the absence and presence of SOD. The oxidized LDL-induced renin release was strongly inhibited by catalase and completely prevented in the presence of both catalase and SOD [38]. These findings indicate that oxidized LDL and LpA are stimulants of renin release by a mechanism that involves the formation of ROS [36, 38]. This conclusion was further reinforced by the observation that high-density lipoprotein (HDL) prevents the stimulatory effect of oxidized lipoproteins on renin release and $O_2^{\bullet-}$ in JG cells [36], which is in accordance to the now well-established antioxidant activity of HDL [39].

Recent evidence also indicates that ROS promote renin release. In primary cultures of mouse JG cells, the exposure for 60 min to an $O_2^{\bullet-}$-generating reaction mixture with hypoxanthine and XOD significantly increased renin release [40]. Tempol prevented this stimulatory effect but did not change basal renin release [40]. Furthermore, the incubation with exogenous H_2O_2 for 60 min enhanced the renin release rate and treatment of JG cells with catalase reduced the basal renin release rate by 45%. These results indicate that ROS such as $O_2^{\bullet-}$ and H_2O_2 can acutely stimulate renin release [40]. Further work by the same group showed that this effect of H_2O_2 on renin release is most likely mediated by cyclic adenosine monophosphate (cAMP) [41]. Moreover, since the NADPH oxidase isoform (Nox) 4 was shown to be expressed in JG cells and silencing of this isoform resulted in a significant reduction of renin release, it was

suggested that endogenously Nox4-derived H_2O_2 in JG cells promotes renin release [42]. *In vivo* experiments were also performed in mice to test the hypothesis that the augmentation of H_2O_2 in the renal cortex stimulates renin release and increases blood pressure. A subcapsular renal catheter connected to an osmotic mini pump to achieve a concentration of 1 μM H_2O_2 was implanted in mice. Two days after the infusion, the systolic blood pressure, measured by radiotelemetry, was shown to be increased by 22 ± 2 mmHg and there was a twofold increase in plasma renin concentration [42]. Overall, these results indicate that renal cortical ROS might contribute to arterial hypertension by increasing renin release [40, 42]. In addition, increased ROS generation appears to reverse the inhibitory influence of other hormones on renin release [43]. Leptin, an adipocyte-derived hormone, exhibits natriuretic effects on normotensive, nonobese animals [43, 44]. However, the natriuretic response to the infusion of leptin appears to be attenuated in animal models of arterial hypertension or obesity [43–45], which are known to be associated with oxidative stress [17, 46–49]. Since the infusion of leptin tends to elevate blood pressure and increased renin levels might contribute to this effect [43, 50], experiments were performed to evaluate the effects of leptin on renin release, under normal conditions or during high oxidative stress [43]. It was observed that leptin treatment for 1 hour reduced renin release in JG cells. However, in cells pretreated with H_2O_2, leptin significantly promoted renin release [43]. These results suggest that increased ROS levels change the impact of leptin on renin release [43] and are in accordance with previous observations that plasma renin activity is positively correlated with systemic leptin concentration in hypertension [51, 52].

In physiological conditions, renin expression and release are under a negative feedback in response to Ang II, macula densa sodium chloride concentration and renal perfusion pressure [26, 53]. The cytokine TNF-α was shown to mediate the drinking and pressor responses to Ang II and to markedly inhibit renin expression [54–56]. Since TNF-α can increase ROS generation and contribute to oxidative stress [57, 58], Itani et al. using an *in vitro* model of JG cells (As4.1 cells) tested the hypothesis that TNF-α increases the production of ROS which in turn inhibit renin mRNA expression [54]. They observed that treatment with TNF-α increased the production of both $O_2^{\bullet-}$ and H_2O_2 in these cells and that NAC reduced the H_2O_2 generation induced by TNF-α [54]. NAC itself had no effect on renin mRNA expression but prevented its attenuation in cells treated with TNF-α [54]. Moreover, H_2O_2 was found to negatively regulate renin mRNA expression and the renin-promoter activity through a mechanism independent of NF-κB activation [54].

The *in vivo* effects of antioxidants or inhibitors of ROS production on renin expression and activity have also been studied in animal models of hypertension. In order to test the hypothesis that in hypertension the increased ROS generation modifies type 1 nitric oxide synthase (NOS1) and cyclooxygenase-2 (COX-2) expression in the JG apparatus, thereby altering renin synthesis and secretion, the NADPH oxidase inhibitor apocynin was given for 3–7-week old Wistar-Kyoto (WKY) and SHR rats [59]. Untreated SHR rats exhibited higher oxidative stress and NOS1 immunoreactivity and lower COX-2 immunoreactivity, renin mRNA expression, renin immunoreactivity and plasma renin activity than the untreated WKY rats [59]. Apocynin treatment reduced oxidative stress and the immunoreactivity of NOS1 and renin in JG apparatus but did not alter COX-2 immunoreactivity, renin mRNA expression, or plasma renin activity in SHR rats and was devoid of effects on all these parameters in WKY rats [59]. These

results suggest that the increased ROS generation in SHR is responsible for the induction of NOS1 expression and augmented nitric oxide (NO) synthesis, thereby increasing local renin expression. Indeed, NO appears to be involved not only in the stimulation of renin secretion but also in the recruitment of renin-expressing cells [60, 61]. Another study in SHR rats evaluated if the antihypertensive response to tempol is related to a decrease in plasma renin activity and in the urinary excretion of isoprostanes, NO metabolites, ET-1, or catecholamines [62]. Tempol administered for 12 days reduced the urinary excretion of isoprostanes, doubled the plasma renin activity and did not alter the urinary excretion of ET-1, NO metabolites, or catecholamines [62]. Although these authors suggested that the increase in plasma renin activity with tempol was due to the decrease in blood pressure [62], the putative contribution of H_2O_2 to this effect in plasma renin activity should be also considered. As a SOD mimetic, tempol converts $O_2^{\bullet-}$ into H_2O_2 and previous studies have shown that increased H_2O_2 production counteracts the putative protective effects of tempol in hypertension [48, 49, 63].

The effects of a lower dose of tempol on renin activity and expression were also investigated in SHR rats fed a high-fat diet. Tempol was given to 8-week old SHR rats fed a high-fat diet for 12 weeks [21]. The administration of high-fat diet was associated with increased systolic blood pressure, unaltered plasma renin activity, increased oxidative stress and reduced urinary excretion of NO metabolites in SHR [21]. Furthermore, these rats also exhibited increases in the JG renin immunoreactivity and in the renal cortical mRNA and protein expression of renin [21]. Treatment with tempol reduced oxidative stress, improved the urinary excretion of NO metabolites, did not alter plasma renin activity, but significantly reduced the impact of the high-fat diet on the other renin parameters evaluated in that study [21]. Thus, increased $O_2^{\bullet-}$ production appears to enhance intrarenal renin expression in SHR rats fed a high-fat diet. In contrast, no changes were observed in renin expression or immunoreactivity in SHR fed a normal-fat diet or in WKY rats fed a normal or a high-fat diet [21].

In addition to the studies demonstrating a role for ROS in the regulation of renin expression and release, there is also evidence that PRR is upregulated in conditions of enhanced ROS generation. In STZ-induced diabetic Sprague-Dawley rats, the renal mRNA and protein expression of PRR, as well as the PRR immunostaining in glomeruli and tubules, were significantly increased compared to control rats [64]. Treatment of STZ-diabetic rats with DPI or with the Ang II type 1 (AT$_1$) receptor blocker valsartan for 1 week prevented the increases in renal PRR mRNA, protein and immunoreactivity [64]. These results indicate that in diabetes the upregulation of renal PRR results from the activation of both AT$_1$ receptor and the ROS-generating NADPH oxidase [64].

The modulation of PRR expression by ROS was also studied in a model of enhanced ROS generation induced by the deletion of DJ-1, a multifunctional antioxidant protein that scavenges ROS and also regulates the expression of several genes by directly interacting with histone deacetylase [65–69]. DJ-1-knockout mice (DJ-1$^{-/-}$) had increased renal mRNA, protein and immunoreactivity of PRR, increased ERK1/2 activation in response to prorenin and increased fibrotic gene expression compared to the WT animals (DJ-1$^{+/+}$) [66]. A decreased histone deacetylase 1 recruitment at the PRR promoter and a reduction of its histone acetylation were also observed in DJ-1$^{-/-}$ mice [66]. Furthermore, mesangial cells derived from DJ-1$^{-/-}$ mice

animals exhibited increased H_2O_2 generation compared with those from DJ-1$^{+/+}$ mice [66]. The effects on PRR expression and epigenetic regulation were induced by the treatment with H_2O_2 and reversed by the addition of the antioxidant NAC in DJ-1$^{+/+}$ mesangial cells. Furthermore, silencing of PRR by transfecting mesangial cells with siRNA-PRR markedly reduced the expression of fibrotic genes [66]. Therefore, it was concluded that the reduction of DJ-1 protein might hasten renal damage via H_2O_2-mediated epigenetic regulation of PRR expression [66].

Evidence for the regulation of renin and the (pro)renin receptor by ROS is presented in **Figure 2**.
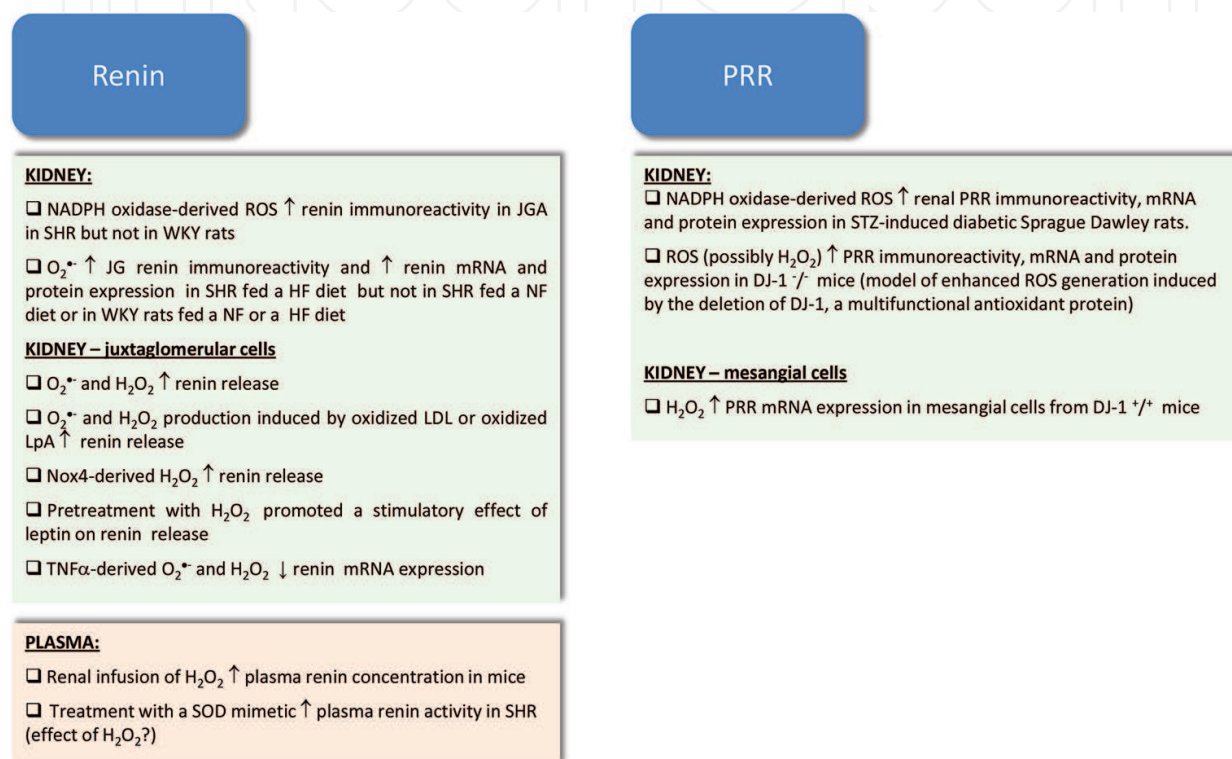
Figure 2. Regulation of renin and pro(renin) receptor by ROS. JG, juxtaglomerular; JGA, juxtaglomerular apparatus; LDL, low-density lipoprotein; LpA, lipoprotein A; PRR, pro (renin) receptor; ROS, reactive oxygen species; SOD, superoxide dismutase; SHR, spontaneously hypertensive rats; STZ, streptozotocin; TNFα, tumoral necrosis factor alpha; WKY, Wistar Kyoto.

2.3. ROS, ACE and ACE2

ACE is a 1306-amino acid 140 kDa zinc-containing metalloprotease that acts as a dipeptidyl carboxypeptidase, hydrolyzing the physiologically inactive decapeptide Ang I to the physiologically active octapeptide Ang II [70], thus being crucial for the formation of the major effector of the RAAS. ACE also inactivates the vasodilator bradykinin [70]. ACE has two catalytic domains: NH$_2$- and COOH-terminus that are highly homologous although the preferential catalytic conditions and the rate of hydrolysis might differ for the same substrate [71]. In 2000, two independent research groups came out with a homologous form of ACE (40–42% homology)—the angiotensin-converting enzyme 2 (ACE2)—which is also a zinc metalloprotease

with carboxypeptidase activity [72, 73]. However, ACE2 is a mono-carboxypeptidase and so, it catalyzes the conversion of Ang I or Ang II to the nonapeptide angiotensin (1–9) [Ang (1–9)] or the heptapeptide angiotensin (1–7) [Ang (1–7)], respectively [72]. As the affinity of ACE2 for Ang II is 400-fold higher than that for Ang I, the formation of Ang (1–7) predominates [74, 75]. The ACE2/Ang (1–7)/MAS axis has been highlighted as the counterregulatory arm of the RAAS [76]. The balance between the activities of ACE and ACE2 will determine, respectively, the relative levels of Ang II and Ang (1–7) at the surface of the correspondent receptors and, thus, the net effect of the RAAS.

The first evidence concerning a putative role of ROS on ACE activity comes from a study of Tominaga et al., in 1988, who observed that the thiol-oxidizing agent diamide markedly increased the activity of ACE in crude extracts of rat renal cortex, heart and brain while causing a moderate increase in ACE activity in the lung and aorta and no alteration in plasma ACE activity [77]. By that time, no particular ROS was identified as being responsible for the reported effect. However, in 1993 Chen and Catravas [78] reported that *in vitro* H_2O_2 or the ROS-generating system XOD decreased the activity of ACE in cultured bovine pulmonary endothelial cells, contrary to what was expected from the results of the pioneering study. Moreover, Chen and Catravas observed that H_2O_2 was also responsible for the decrease in ACE activity when neutrophils were activated with phorbol 12-myristate 13-acetate (PMA) [78]. Indeed, they characterized the effect as being the result of the production of H_2O_2 and its intracellular conversion into $^{\bullet}OH$ through the iron-catalyzed Haber-Weiss reaction since the inhibitory effect of activated neutrophils on ACE activity was prevented by catalase and by a cell-permeable scavenger of $^{\bullet}OH$, an iron-chelator and a thiol reducing agent [78]. These results were confirmed in another study using purified ACE from bovine lungs, which showed that H_2O_2 decreased ACE activity at least in part through the generation of $^{\bullet}OH$ from H_2O_2 since an iron chelator attenuated the effect [79]. When tested directly, $^{\bullet}OH$ decreased ACE activity at high concentrations and this effect was prevented by scavengers of $^{\bullet}OH$ and by thiol-reducing agents, thus suggesting oxidation of the thiol groups of ACE [79]. Interestingly, this study revealed that the inhibitory effect was more marked on the COOH-domain than on the NH_2-domain of ACE [79]. Another *in vitro* study showed that neither $O_2^{\bullet -}$ nor H_2O_2 or $^{\bullet}OH$ altered the activity of purified ACE [80]. In contrast to these studies but in line with the study of Tominaga et al. [77], recently it has been reported that in human umbilical vein endothelial cells, H_2O_2 increased the expression of ACE via the cAMP/protein kinase A (PKA)/cAMP response-element binding pathway, although there was also decreased cell viability due to increased apoptosis [81]. These apparent contradictory results have not raised discussion in the literature. Eventually, they might represent an example of species-dependent effect since the only two studies that reported decreased ROS-mediated ACE activity used bovine or rabbit cells while all the others, concerning mostly rats and mice, reported ROS-mediated increases in ACE expression and activity, as already referred above and will be further presented below. Indeed, NADPH oxidase, SOD, or H_2O_2 have been associated with increased ACE expression and/or activity. Alternatively but less probably, it might be that the $^{\bullet}OH$ would have the opposite effect on ACE than the other ROS, putatively reflecting a fine-tuning regulatory network. The fact that some studies evaluated ACE activity while others quantified ACE expression might also contribute to the apparent controversial data. Unfortunately, not

so many studies have addressed this question and so, further studies are needed in order to fully characterize the role of ROS in regulating ACE expression and/or activity.

A role for NADPH oxidase was evident from a study using rats subjected to unilateral nephrectomy (UNX) subjected to an albumin overload, which show overt proteinuria [82]. These rats have serum ACE activity similar to that found in controls but they show increased expression of ACE (mRNA and protein) in the renal cortex, especially in RPTCs; treatment with apocynin had no effect on serum ACE activity but attenuated the increase in renal ACE expression [82]. In another model of renal damage, it was characterized that kidney cells (the NRK52E line) exposed to albumin activated by advanced oxidation protein products (AOPPs) (usually generated by the reaction of proteins with hypochlorous acid) show increased expression (mRNA and protein) and activity of ACE via activation of cluster of differentiation 36 (CD36) and the receptor for advanced glycation end products and the $PKC\alpha$-NADPH oxidase pathway [83]. Consistently, in Sprague-Dawley rats with UNX and daily intravenous injections of albumin activated by AOPPs for 3 weeks, renal ACE expression (mRNA and protein) and activity increased, mainly in PTCs, although plasma levels and activity of ACE did not change [83]. Treatment with apocynin attenuated the increase in renal ACE expression and activity [83]. Also, DPI prevented the increase in ACE mRNA levels induced by high glucose in the glomerular mesangial cell line HBZY-1 [14].

The above referred study of Chen and Catravas [78] excluded a role of $O_2^{\bullet-}$, hypochlorous acid, peroxynitrite, or proteases in the decrease of ACE activity found in PMA-activated neutrophils from New Zealand rabbits since the effect was not altered by SOD, an MPO inhibitor, hypochlorous acid scavengers, an inhibitor of NO synthesis and proteinases inhibitors. However, tempol abolished the increase in renal ACE mRNA levels observed in SHR rats fed with a high-fat diet in comparison with those in a normal-fat diet [21]. Interestingly, normotensive WKY rats fed a high-fat diet showed the same renal ACE mRNA levels as those normotensive rats on a normal-fat diet [21]. Similarly, obese Zucker rats show higher expression of ACE (mRNA and protein) than lean controls and tempol treatment normalized the differences found in obese Zucker rats [84]. Furthermore, $O_2^{\bullet-}$ has also been implicated in the increased expression of ACE protein in the hypothalamic paraventricular nucleus (PVN) of the Sprague-Dawley rat intravenous infused with Ang II since the effect was attenuated by bilateral microinjections of tempol [85].

The increased expression of ACE protein was also found in the PVN of Sprague-Dawley rats fed a high-salt diet compared with the normal-salt fed rats [86]. Interestingly, bilateral microinjections of PEG-catalase into the PVN attenuated this increase while microinjections of aminotriazole (a catalase inhibitor) augmented it, thus suggesting a role for endogenous H_2O_2 in the regulation of ACE expression [86]. H_2O_2-mediated increase in ACE expression was also reported to occur in the diabetic Akita mice, in which the higher renal ACE expression (mRNA and protein) was normalized by overexpression of catalase in the RPTCs [12].

If a regulatory effect of ROS on ACE expression and/or activity has been the aim of some studies, evidence for an impact of ROS on ACE2 expression and/or activity is still quite scarce. The above referred study on the diabetic Akita mice showed a decrease in the renal ACE2 expression (mRNA and protein) that was normalized by overexpression of catalase specifically in the RPTCs [12]. Curiously, the obese Zucker rats show lower expression of ACE2 (mRNA and protein)

besides higher expression of ACE than the lean controls and these differences were normalized by tempol treatment [84]. Interestingly, overexpression of catalase in the RPTCs [12] or tempol treatment in Zucker lean rats [84] did not alter the expression of either ACE or ACE2. Taken together, these results suggest that endogenous SOD and H_2O_2 might be crucial for the regulation of ACE and ACE2 expression in the context of diabetes although not in the physiological context. Another very recent study focused on the vascular activity of ACE2 through the characterization of the relaxant effect mediated by Ang II on rat carotid rings [87]. In this setup, Ang II caused a biphasic response over a precontraction induced by phenylephrine: a contraction (for nM range Ang II) followed by a relaxation that came to the previous phenylephrine-induced tone and even further to a tension that was below that of the phenylephrine-induced contraction (for μM range Ang II). This second part of the Ang II-mediated relaxation reflects ACE2 activity since it was the only part of the response to Ang II that was blocked by a MAS receptor antagonist and considering that ACE2 is the only enzyme responsible for the conversion of Ang II in Ang (1–7) (the endogenous agonist of the MAS receptor). The authors observed that in control rats, this Ang II-mediated vasorelaxation (reflecting ACE2 activity) was not altered by apocynin, tiron, or PEG-catalase. However, in STZ-diabetic rats, the ACE2/Ang (1–7)/MAS-mediated vasorelaxant effect was usually absent but it was restored by apocynin, tiron and PEG-catalase, suggesting that NADPH oxidase-$O_2^{\bullet-}$-H_2O_2 play a significant role in this effect, namely through ROS-mediated inhibition of ACE2 activity [87].

Figures 3 and **4** summarize the role of ROS in the regulation of ACE and ACE2, respectively.

ACE

KIDNEY, HEART, BRAIN, LUNG, AORTA:
❑ ROS ↑ ACE activity

KIDNEY:
❑ $O_2^{\bullet-}$ ↑ ACE mRNA (SHR rats fed a HF diet and obese Zucker rats) and protein (obese Zucker rats); no effect in control rats
❑ H_2O_2 ↑ ACE mRNA and protein in Akita diabetic mice but not in controls

KIDNEY – proximal tubule cells:
❑ NADPH oxidase-derived ROS ↑ ACE mRNA, protein and activity in AOPPs-stimulated conditions

KIDNEY – glomerular mesangial cells:
❑ NADPH oxidase-derived ROS ↑ ACE mRNA in HG conditions

BRAIN – hypothalamic paraventricular nucleus:
❑ $O_2^{\bullet-}$ ↑ ACE protein in Ang II-hypertensive rats but not in controls
❑ H_2O_2 ↑ ACE protein in animals under HS diet but not under NS diet

PLASMA:
❑ ROS did not alter ACE activity
❑ H_2O_2 ↓ ACE activity in PMA-activated PMN cells
❑ •OH ↓ ACE activity in PMA-activated PMN cells
❑ $O_2^{\bullet-}$, HClO or ONOO⁻ do not alter ACE activity in PMA-activated PMN cells
❑ NADPH oxidase-derived ROS did not alter ACE activity in AOPPs-stimulated conditions

ENDOTHELIAL CELLS:
❑ H_2O_2 ↓ ACE activity in PMA-activated PMN cells
❑ ROS ↓ ACE activity in PMA-activated PMN cells
❑ H_2O_2 ↑ ACE expression in HUVEC

IN VITRO:
❑ H_2O_2 ↓ ACE activity in vitro
❑ •OH ↓ ACE activity in vitro
❑ ROS do not alter ACE activity in vitro
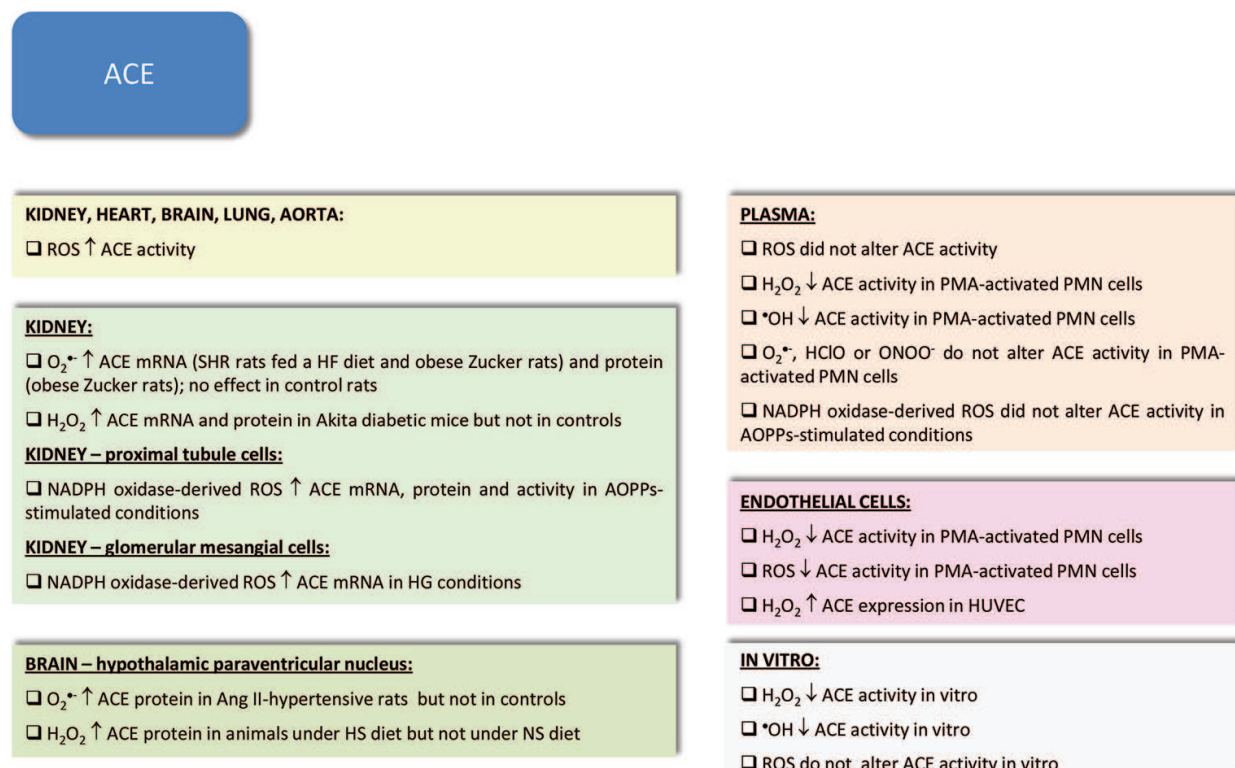
Figure 3. Regulation of ACE by ROS. ACE, angiotensin converting enzyme; AngII, angiotensin II; AOPPs, advanced oxidation protein products; HF, high fat; HG, high glucose; HS, high salt; HUVEC, human umbilical vein endothelial cells; NS, normal salt; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophils; ROS, reactive oxygen species; SHR, Spontaneously Hypertensive Rats.
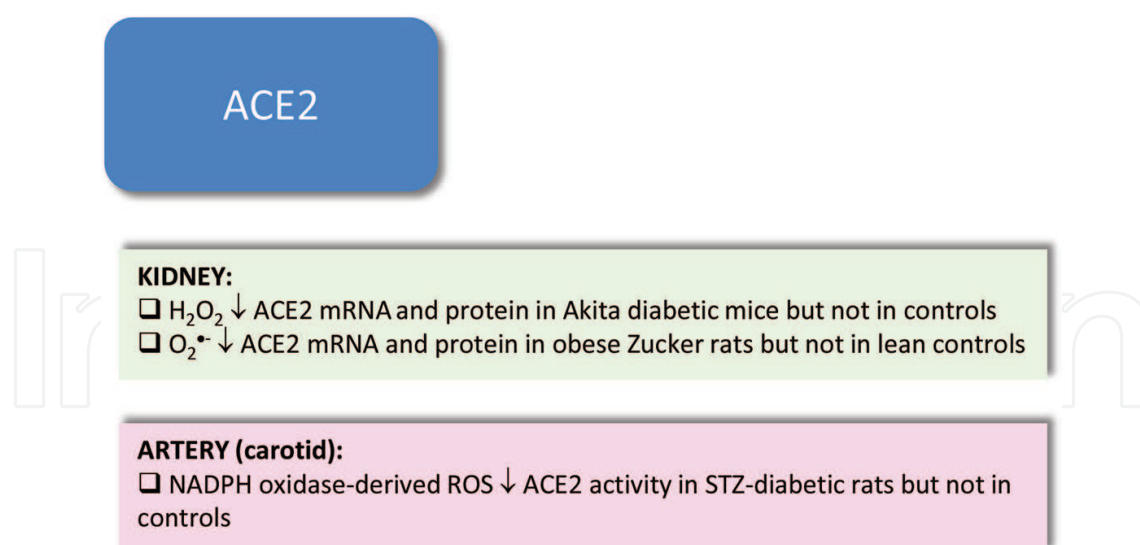
Figure 4. Regulation of ACE2 by ROS. ACE2, angiotensin converting enzyme 2; ROS, reactive oxygen species; STZ, streptozotocin.

2.4. ROS, Ang II, Ang (1–7) and aldosterone

Ang II, the most important peptide of the RAAS, is mainly formed from the precursor AGT by the sequential action of renin and ACE. Other angiotensin-derived peptides also exhibit biological activity, including angiotensin 2–8 (Ang III), angiotensin 3–8 (Ang IV) and Ang (1–7). Ang III and Ang IV are products from the catabolism of Ang II at the NH_2-terminus by aminopeptidases A and N. In human tissues there are several alternative ACE-independent pathways for Ang II formation, including proteinases such as chymase, kallikrein, cathepsin G and elastase-2 whose clinical significance is not yet explored [88, 89]. Ang II binds to two receptor subtypes, the AT_1 and Ang II type 2 (AT_2) receptors, that belong to the G-protein-coupled receptor (GPCR) family but differ in terms of tissue distribution and cell signaling pathways. Most of the known vasoactive, mitogenic, proinflammatory and profibrotic effects of Ang II are mediated by the activation of AT_1 receptor, but it can also bind to the AT_2 receptor thereby triggering opposite effects to those elicited by the AT_1 receptor [90–92]. Importantly, Ang II-AT_1 receptor interaction stimulates the activation of NADPH oxidase, a major source of ROS in the heart, vasculature, kidneys and central nervous system [93]. Under pathological conditions, characterized by RAAS activation, such as arterial hypertension, diabetes, atherosclerosis and heart failure, there is an Ang II-induced increase in the expression and/or activity of several Nox, leading to higher ROS generation and oxidative stress [93–100]. Ang (1–7), an active peptide of this system that typically opposes the effects of Ang II in the cardiovascular system, is formed primarily from Ang II through the action of ACE2 at the COOH-terminus but may also be formed by the cleavage of Ang I by neutral endopeptidases [28, 101, 102]. Many of Ang (1–7) counteracting actions on AT_1 receptor-mediated effects occur via the MAS receptor. However, this peptide may also interact with AT_2 and AT_1 receptors. Ang (1–7) seems to play a protective role in cardiometabolic and renal diseases due to its antihypertensive, antiproliferative, antifibrotic, antiarrhytmic, antithrombotic, antidiabetic, natriuretic and diuretic effects [28, 101–105]. Moreover, it also has antioxidant and anti-inflammatory actions [106–108].

Interestingly, both Ang II and Ang (1–7) content appear to be modulated by ROS. In cardiac fibroblasts from young adult male Sprague Dawley rats, treatment with H_2O_2 for 3 hours caused a threefold increase in secreted Ang II levels [22]. Oxidative stress induced by *in vitro* or *in vivo* treatment with high concentrations of albumin or with AOPP-modified albumin also resulted in increased Ang II levels in cultured RPTCs or in the renal cortex of UNX rats [82, 83]. Noteworthy, treatment with apocynin reduced the Ang II content in the renal cortex of UNX rats subjected to high concentrations of albumin or AOPP-modified albumin [82, 83]. In SHR fed a high-fat diet for 12 weeks there was also an increase in renal immunoreactivity and concentration of Ang II which was counteracted by tempol treatment but no changes were observed in SHR fed a normal-fat diet or in WKY fed a normal- or a high-fat diet [21]. Obese Zucker rats exhibited a similar concentration of Ang II and reduced Ang (1–7) content in the renal cortex, as compared to lean Zucker rats. Obese rats had also increased diuretic and natriuretic responses to AT_1 receptor blockade and decreased natriuretic response to Ang (1–7). In obese Zucker rats, but not in lean controls, treatment with tempol significantly decreased renal cortical Ang II content, augmented Ang (1–7) concentration and reverted the increase in AT_1 receptor-mediated effect and the decrease in the natriuretic response to Ang (1–7) [84]. Moreover, the enhanced Ang II immunostaining observed in proximal convoluted tubules and cortical collecting ducts of Sprague Dawley rats subjected to acute sodium overload was also normalized by tempol treatment. The concomitant decrease of hypoxia-inducible factor 1α and increase of eNOS expression induced by tempol administration to these rats suggest oxidative stress inhibition [109]. Type 1 diabetic Akita mice had unchanged serum Ang II concentration, higher urinary Ang II levels and lower urinary content of Ang (1–7) compared to nonAkita WT mice. Renal mRNA and protein expression of ACE and ACE2 in Akita mice followed a similar pattern to that observed for urinary Ang II and urinary Ang (1–7), respectively. The overexpression of catalase in RPTCs of Akita mice did not alter serum Ang II levels but reduced the renal ACE expression and urinary Ang II content and normalized renal expression of ACE2 and urinary Ang (1–7) levels [12]. Additionally, in cultured rat mesangial cells, treatment with high glucose induced an increase in ROS generation, as well as an elevation in the mRNA expression of AGT, ACE and AT_1 receptor and in Ang II concentration in the media. Incubation with DPI reduced ROS generation and the mRNA expression of RAAS components in these cells [14]. The exposure of cultured vascular smooth muscle cells (VSMCs) to high-glucose media significantly decreased Ang (1–7) concentration in cell lysates compared to that observed under normal-glucose conditions. High glucose also induced an upregulation of Nox1 mRNA and protein expression, while decreasing the expression of Nox4. Treatment with DPI, apocynin, or catalase reverted the lowering effect of high-glucose on Ang (1–7) content but caused a significant reduction of Ang (1–7) in cells exposed to normal-glucose media. These results suggest that high glucose stimulates the production of Nox1-derived ROS that causes a reduction in Ang 1–7 content. In contrast, under normal glucose conditions, Nox1- or Nox4-derived ROS appear to contribute to maintain the physiological concentrations of Ang (1–7) [110]. The changes in Ang II or Ang (1–7) content observed in these studies probably result from the ROS modulation of renin, AGT, ACE, or ACE2, although for Ang II we cannot exclude an effect of ROS on other alternative pathways responsible for its production.

Aldosterone is a steroid hormone primarily produced and secreted by zona glomerulosa in the adrenal cortex in response to Ang II stimulation through the AT_1 receptor [111]. Its synthesis

from cholesterol involves a series of hydroxylation and oxidation reactions by members of the cytochrome P450 super family such as aldosterone synthase (CYP11B2), the key enzyme that catalyzes the final step of aldosterone synthesis and is excessively produced in the type 1 form of familial primary aldosteronism (PA) [112]. Patients with PA exhibit an increased susceptibility to cardiovascular complications, including left ventricular hypertrophy, stroke, nonfatal myocardial infarction, atrial fibrillation, as well as higher levels of oxidative stress markers than essential hypertensive patients, which decrease after specific treatment of PA [113, 114]. Noteworthy, ROS seem to be upstream regulators of aldosterone synthesis. In a study performed in human and rat adrenal cortical cells, Ang II increased CYP11B2 activity, mRNA and protein with simultaneous elevation of oxidative stress by-products, NADPH oxidase activity and H_2O_2 levels. These Ang II-induced effects were abolished or attenuated by pretreatment of cells with either the AT_1 receptor antagonist losartan, the antioxidants PEG-catalase and NAC, the Nox inhibitor VAS-2870, siRNA silencing of Nox1, 2 and 4, or inhibitors of phospholipase C (PLC) and PKC. Importantly, treatment with H_2O_2 mimicked the facilitatory effects of Ang II on CYP11B2 activity, mRNA and protein expression and these changes were absent or attenuated in PEG-catalase pretreated cells, suggesting that H_2O_2 is a key regulator of aldosterone production [115].

Plasma aldosterone levels were also shown to be modulated by the induction of heme oxygenase-1 (HO-1), an important antioxidant pathway [116–118]. In a rat model of renovascular hypertension treatment with cobalt protoporphyrin markedly increased the expression and activity of HO-1 and these effects were accompanied by a marked attenuation of the development of hypertension, decreased oxidative stress and reduced plasma aldosterone concentration [116]. Although the mechanisms contributing to a lower aldosterone synthesis by HO-1 induction remain to be clarified, the authors speculated that HO-1 might inhibit the CYP450 enzymes required for aldosterone formation, by limiting the availability of heme or by increasing the production of carbon monoxide [116]. Of note, heme is a prooxidant molecule that has been shown to contribute to increased generation of ROS and lipid peroxidation, while the HO-1 product carbon monoxide appears to possess antioxidant properties [116, 118].

Secretory products derived from visceral adipocytes have also been shown to upregulate aldosterone synthase expression and stimulate adrenal aldosterone synthesis thus suggesting a direct link between obesity and hypertension [119–122]. In fact, several clinical studies have already observed elevated plasma aldosterone levels in obese patients [121, 122]. In an experimental model of obesity, it was also shown that the enhanced blood pressure response to Ang II was associated with an increase in circulating aldosterone. Ang II infusion induced a more prominent increase in plasma aldosterone levels and blood pressure in obese Zucker rats that in lean controls. These results corroborate the hypothesis that aldosterone contributes to obesity-related hypertension [123]. Furthermore, even though the basal circulating aldosterone concentration was similar in lean and obese Zucker rats [84, 123], treatment with tempol significantly reduced serum aldosterone levels, in addition to its antioxidant and blood pressure lowering effects, in obese but not in lean Zucker rats [84]. Of note, although the link between obesity and increased systemic aldosterone concentration has not been consistently evidenced, it has been reported that

in obesity-induced hypertension an intrarenal RAAS might operate independently of the systemic RAAS, contributing to increased aldosterone action [21, 124]. Chung et al. demonstrated that SHR fed a high-fat diet for 12 weeks exhibited increased renal cortical expression of several RAAS components and augmented 24-h urinary excretion of aldosterone, despite the absence of changes in plasma renin activity or plasma aldosterone concentration. These SHR rats had also higher blood pressure and renal oxidative stress, as well as lower fractional excretion of sodium, than those maintained on a normal-fat diet. Importantly, tempol significantly attenuated the high-fat diet-induced increases in the renal expression of RAAS components and in urinary aldosterone excretion and blunted or attenuated the changes in oxidative stress, blood pressure and sodium reabsorption [21]. These findings emphasize the importance of ROS as regulators of renal RAAS components, including aldosterone and suggest that the use of a SOD mimetic might be an effective therapy to prevent the progression of hypertension in obese subjects. Indeed, in the remnant kidney rat model, an experimental model of progressive nephropathy, treatment with NAC had a protective effect also attributable to a decrease in oxidative stress and plasma aldosterone levels [125]. The beneficial effect that NAC had on glomerular filtration rate was more impressive than the modest reduction in proteinuria and was independent of blood pressure reduction [125]. Additionally, the combination of NAC and spironolactone was found to confer additive protection in the same model, improving blood pressure control and renal function more than did NAC or spironolactone alone, thus suggesting that antioxidant/antihypertensive combinations could be important therapeutic strategies to attenuate the aggravation of chronic renal disease [125].

The classical genomic pathway whereby aldosterone exerts its effects involves the binding to the cytosolic mineralocorticoid receptor (MR) within the renal cortical collecting duct cells and subsequent translocation of this aldosterone-MR complex to the nucleus, thereby promoting the transcription of genes that regulate electrolyte and fluid balance resulting in sodium reabsorption, water retention and potassium and magnesium loss, with consequent volume expansion and blood pressure rise [102]. It is well known now that inappropriate regulation of the aldosterone/MR system contributes to sodium retention and hypertension and to the development of renal injury [126]. These adverse actions of aldosterone in the kidney appear to involve the production of ROS that activate the MAPK pathway in renal cortical tissues, which in turn causes renal injury [127, 128]. Interestingly, MR activation and subsequent renal injury may be triggered by other ligands and/or pathological conditions besides aldosterone [129]. In Dahl salt-sensitive rats, glomerular MR was activated by high-salt-feeding-induced oxidative stress and this effect was suppressed by tempol. *In vitro* luciferase assays also confirmed that oxidative stress can accelerate MR transcriptional activity in the glomeruli cells [129]. Moreover, MR activation was sustained by high ROS production even after reducing salt intake. Therefore, oxidative stress appears to limit the therapeutic effects of salt restriction, an important therapeutic strategy for salt-sensitive hypertensive patients [129]. Since previous studies also demonstrated that ROS stimulate aldosterone production [21, 115, 125], the use of antioxidants might be an effective strategy to protect the kidney from the overactivation of the aldosterone/MR system.

The main effects of ROS on AngII, Ang (1–7) and aldosterone are depicted in **Figures 5** and **6**, respectively.

Ang II

KIDNEY:

❏ NADPH oxidase-derived ROS ↑ Ang II in the renal cortex of UNX rats (models of hypertension and renal injury)

❏ $O_2^{\bullet-}$ ↑ renal Ang II immunoreactivity and concentration in SHR fed a HF diet (but not in SHR fed a NF diet or in WKY fed a HF or a NF diet)

❏ Reduction of $O_2^{\bullet-}$ ↓ renal cortical Ang II content in obese Zucker rats but not in lean controls

❏ $O_2^{\bullet-}$ ↑ renal Ang II concentration in Sprague Dawley rats subjected to sodium overload

KIDNEY – glomerular mesangial cells:

❏ ↑ ROS associated to ↑ AngII concentration in the media under HG conditions

HEART – fibroblasts:

❏ H_2O_2 ↑ Ang II concentration

SERUM:

❏ H_2O_2 did not alter serum Ang II concentration in type 1 diabetic Akita mice

URINE:

❏ H_2O_2 ↑ urinary Ang II levels in type 1 diabetic Akita mice

Ang (1-7)

KIDNEY:

❏ $O_2^{\bullet-}$ ↓ renal cortical Ang (1-7) content and the natriuretic response to Ang (1-7) in obese Zucker rats but not in lean rats

ARTERIES - VSMCs:

❏ Nox1-derived ROS ↓ Ang (1-7) content under HG conditions

❏ Nox1- or Nox4-derived ROS contribute to the maintenance of physiological Ang (1-7) concentration under NG conditions

URINE:

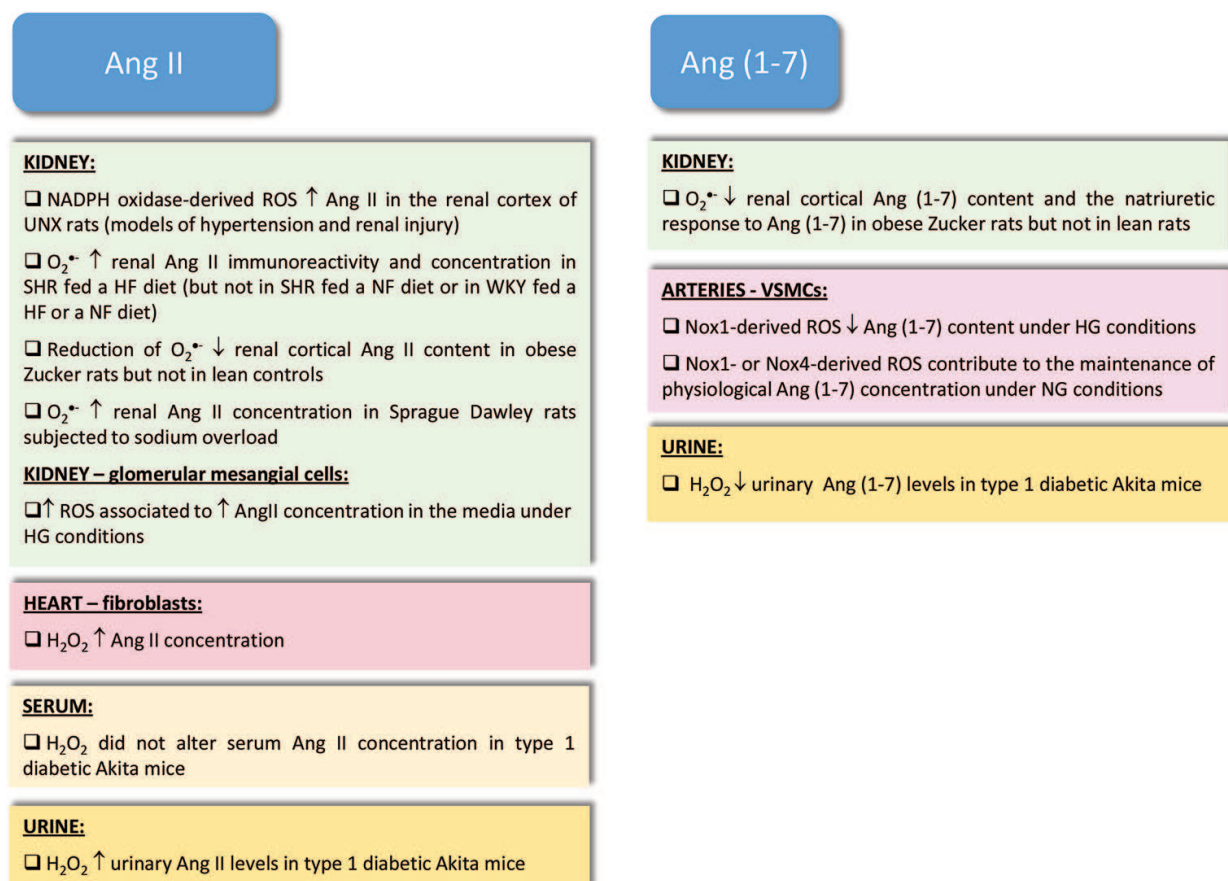❏ H_2O_2 ↓ urinary Ang (1-7) levels in type 1 diabetic Akita mice

Figure 5. Regulation of AngII and Ang (1–7) by ROS. Ang (1–7), angiotensin 1–7; Ang II, angiotensin II; HF, high-fat; HG, high-glucose; NF, normal-fat; ROS, reactive oxygen species; SHR, Spontaneously Hypertensive Rats; UNX, uninephrectomized; WKY, Wistar Kyoto.
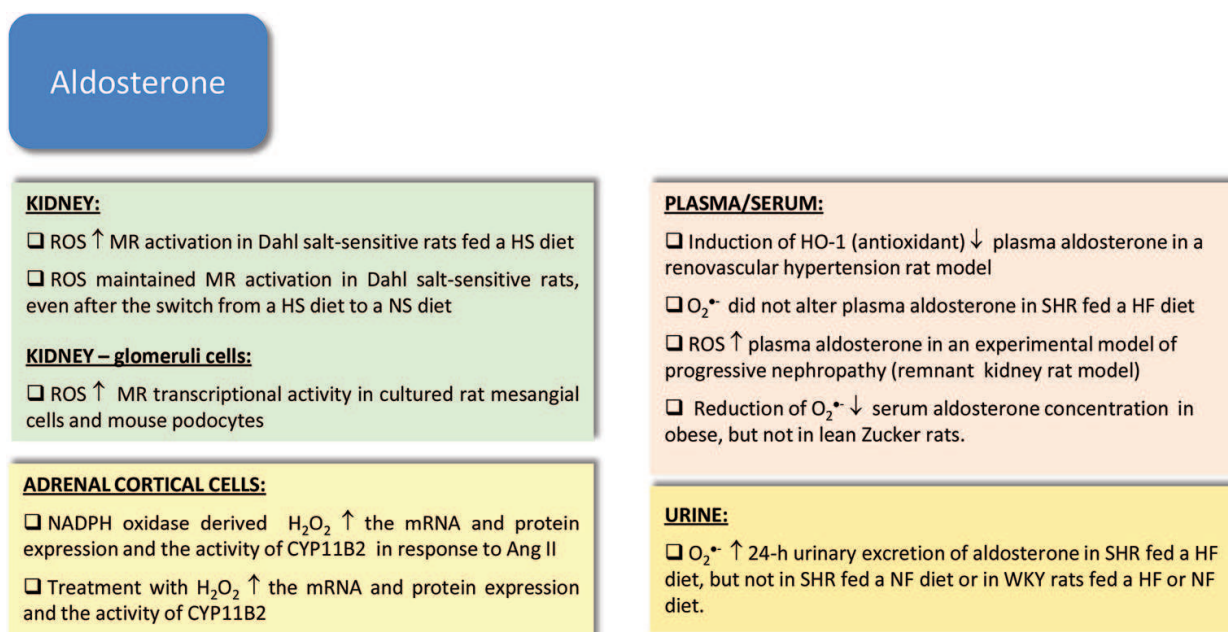
Aldosterone

KIDNEY:

❏ ROS ↑ MR activation in Dahl salt-sensitive rats fed a HS diet

❏ ROS maintained MR activation in Dahl salt-sensitive rats, even after the switch from a HS diet to a NS diet

KIDNEY – glomeruli cells:

❏ ROS ↑ MR transcriptional activity in cultured rat mesangial cells and mouse podocytes

ADRENAL CORTICAL CELLS:

❏ NADPH oxidase derived H_2O_2 ↑ the mRNA and protein expression and the activity of CYP11B2 in response to Ang II

❏ Treatment with H_2O_2 ↑ the mRNA and protein expression and the activity of CYP11B2

PLASMA/SERUM:

❏ Induction of HO-1 (antioxidant) ↓ plasma aldosterone in a renovascular hypertension rat model

❏ $O_2^{\bullet-}$ did not alter plasma aldosterone in SHR fed a HF diet

❏ ROS ↑ plasma aldosterone in an experimental model of progressive nephropathy (remnant kidney rat model)

❏ Reduction of $O_2^{\bullet-}$ ↓ serum aldosterone concentration in obese, but not in lean Zucker rats.

URINE:

❏ $O_2^{\bullet-}$ ↑ 24-h urinary excretion of aldosterone in SHR fed a HF diet, but not in SHR fed a NF diet or in WKY rats fed a HF or NF diet.

Figure 6. Regulation of aldosterone by ROS. Ang II, angiotensin II; HF, high-fat; HO-1, heme oxygenase-1; HS, high-salt; MR, mineralocorticoid receptor; NF, normal-fat; NS, normal-salt; ROS, reactive oxygen species; SHR, Spontaneously Hypertensive Rats; WKY, Wistar Kyoto.

2.5. ROS and Ang receptors

Ang II, the major effector of the RAAS, elicits its actions by binding to the AT_1 or to the AT_2 receptor, which belong to the GPCR superfamily [91]. The AT_1 receptor actually comprises two isoforms, the AT_{1A} receptor and AT_{1B} receptor subtypes that share 95% amino acid sequence homology. Although they have been considered pharmacologically identical, there appears to be differences in their tissue distribution and transcriptional regulation [91]. Furthermore, several studies have suggested that in vascular tissues, AT_1 receptors are AT_{1B} prejunctionally and AT_{1A} postjunctionally [130–133]. AT_1 receptor activation initiates several signaling pathways, including those associated with heterotrimeric G-proteins, G-protein independent β-arrestin, nonreceptor and receptor tyrosine kinases, ROS and small guanosine triphosphate (GTP) binding proteins, which contribute for the wide range of responses to Ang II [91]. One important feature of the AT_1 receptor is the rapid phosphorylation and internalization that occur following stimulation by Ang II [91, 134]. This physiological mechanism limits the functional availability of AT_1 receptors on the cell surface, thus avoiding exaggerated responsiveness to Ang II [134]. Several physiological and pathological factors, including Ang II, ROS, cytokines, growth factors and hormones, regulate AT_1 receptors in all organs [91, 134, 135].

The AT_2 receptor shares only 34% amino acid sequence homology with the AT_1 receptor and exhibits obvious differences in its tissue-specific expression, signaling pathways, pharmacological features and regulation of receptor function [91]. Signal transduction mechanisms initiated by AT_2 receptor activation are unusual for a GPCR and markedly different from those driven by AT_1 receptor. Of note, the AT_2 receptor does not undergo desensitization and internalization on stimulation by Ang II [91]. The AT_2 receptor signaling involves G_i/G_o activation, protein phosphatases, scaffold proteins, NO/cyclic guanosine monophosphate (cGMP), ion channel protein and constitutive activity (ligand-independent actions). The AT_2 receptor is expressed in low levels in normal nongrowing cells [91].

There is evidence that AT_1 and AT_2 receptors mediate opposite actions in response to Ang II. AT_1 receptor activation induces several effects such as vasoconstriction, enhancement of sympathetic outflow, aldosterone release, sodium reabsorption, ROS generation, inflammation, cell proliferation and extracellular matrix formation that contribute to cardiovascular and renal dysfunction under conditions of enhanced AT_1 receptor stimulation [90, 91]. In contrast, AT_2 receptor appears to play a beneficial role in cardiovascular disease due to its vasodilatory, natriuretic, apoptotic, anti-proliferative, antifibrotic and anti-inflammatory effects [90, 91, 136]. Of note, some of these AT_2 receptor actions appear to be best detected under partial AT_1 receptor blockade [91, 102]. Given the protective effects of AT_2 receptor activation, research is being conducted in order to develop specific agonists of AT_2 receptors [102]. The compound 21 is one of these drugs, but unexpectedly it had no effect or even increased blood pressure, an effect that may be related to the fact that in SHR the AT_2 receptors may present an AT_1 receptor-like profile [102, 137]. Nevertheless, AT_2 receptor agonists may be useful to protect against tissue injury [102, 136].

ACE2 transforms Ang II into Ang (1–7), which has been shown to exert vasodilatory, antiproliferative, natriuretic, antithrombotic and antiarrhytmic actions. The MAS receptor, an orphan GPCR, appears to mediate many of these effects and has therefore been proposed as a candidate receptor for this RAAS peptide [91]. Indeed, MAS-knockout mice exhibit changes in heart rate and blood pressure variability, impaired cardiac and renal function accompanied by

profibrotic changes, increased expression of proinflammatory molecules and several metabolic changes such as augmented abdominal fat mass, dyslipidemia, increased insulin and leptin concentration and altered response of adipocytes to insulin [91]. Nevertheless, deletion of the MAS gene may confer protection against salt-induced hypertension and cardiac or renal ischemia-reperfusion injury [91]. Activation of MAS receptor by Ang (1–7) is thought to involve the production of arachidonic acid and nitric oxide synthase (NOS) activation. The potential protective effects of MAS activation by Ang (1–7) make this receptor an attractive drug target [91].

The majority of studies evaluating the regulation of AT_1 receptors by ROS has been performed in the kidney and has demonstrated a stimulating effect of these species on AT_1 receptors [14, 17, 21, 82–84, 138–141]. In adult male Sprague Dawley rats treated for 2 or 3 weeks with L-buthionine sulfoximine (BSO), a prooxidant agent that inhibits the synthesis of glutathione (GSH) [142], the increase in oxidative stress and blood pressure was accompanied by the upregulation of the mRNA, protein and ligand binding of the AT_1 receptor in renal proximal tubules when compared to normotensive controls [138, 139]. Furthermore, incubation with Ang II had a markedly higher impact on AT_1 receptor signaling and on the activation of the sodium transporters Na^+/K^+-ATPase and Na^+/H^+ exchanger 3 in renal proximal tubules from BSO-treated rats than in those from control rats [138, 139]. Treatment for 2 or 3 weeks with tempol decreased oxidative stress and normalized AT_1 receptor mRNA, protein and ligand binding [138, 139]. Furthermore, tempol also reduced AT_1 receptor signaling and activation of sodium transporters in response to Ang II [138, 139]. Overall, the restoration of AT_1 expression and signaling with the antioxidant tempol might have contributed to the normalization of blood pressure in BSO-treated rats [138, 139]. The protective effects of tempol on AT_1 receptor regulation were also evidenced in obese Zucker rats and in SHR fed a high-fat diet [21, 84]. Obese Zucker rats showed higher basal blood pressure values than age-matched lean Zucker rats, as well as an age-dependent increase in blood pressure that was not observed in lean rats [84]. Obese rats also exhibited increased systemic and renal cortical oxidative stress, augmented AT_1-receptor-mediated effects on sodium and water excretion and increased renal cortical mRNA and protein expression of the AT_1 receptor [84]. Tempol treatment for 4 weeks prevented the age-dependent increase in blood pressure in obese Zucker rats, although their blood pressure values remained higher than in lean Zucker rats [84]. Tempol also ameliorated oxidative stress, reversed the AT_1-receptor-mediated actions on sodium and water excretion and decreased the renal cortical mRNA and protein expression of AT_1 receptor in obese Zucker rats but did not alter these parameters in lean Zucker rats [84]. Data from *in vitro* assays were also in agreement with the *in vivo* findings. RPTCs from 14-week-old obese Zucker rats, compared to those from lean Zucker rats, showed a higher protein expression of the AT_1 receptor which was normalized by the *in vitro* treatment with tempol for 24 hours [84]. A significantly higher renal cortical protein expression of AT_1 receptor was also observed in SHR fed a high-fat diet for 12 weeks, starting at the age of 8 weeks. This effect was not verified in SHR fed a normal-fat diet or in WKY rats fed a normal- or a high-fat diet for the same period of time [21]. Furthermore, in SHR fed a high-fat diet and simultaneously treated with tempol, there was a significant reduction in renal cortical AT_1 receptor protein expression [21]. Beneficial effects of tempol have also been demonstrated in a rat aging model [140]. Aged (21 months old) Fischer 344 Brown Norway F1 (FBN) rats exhibited increased oxidative stress, evidenced by the augmented plasma isoprostanes

concentration, decreased urinary antioxidant capacity and increased expression of NADPH oxidase-gp91phox in renal proximal tubular homogenate, when compared to adult (3 months old) FBN rats [140]. These effects were accompanied by exaggerated AT_1 receptor-mediated actions on urine flow and urinary sodium excretion [140]. Tempol treatment for 3 or 4 weeks reduced oxidative stress and normalized the AT_1 receptor-mediated effects on diuresis and urinary sodium excretion in aged but not in adult FBN rats [140].

The impact of ROS on AT_1 receptor regulation has also been studied in *in vivo* or *in vitro* models of diabetes [14, 141]. In STZ-induced diabetic male Sprague Dawley rats, treatment with recombinant human extracellular SOD for 4 weeks, beginning 2 weeks after STZ, prevented the decrease in renal SOD activity and the increase in protein expression of the renal AT_1 receptor induced by STZ intraperitoneal injection [141]. In the rat glomerular mesangial cell line HBZY-1 exposed to a high-glucose medium, ROS generation and the AT_1 receptor mRNA levels were significantly augmented when compared to the effects observed in cells cultured in the normal-glucose medium [14]. These effects were abolished by DPI or by application of NaHS, a donor of the gas transmitter hydrogen sulfide which is also known to exhibit antioxidative properties [14, 143]. Intriguingly, in the same study these authors observed a downregulation, instead of an upregulation, of the AT_1 receptor mRNA expression in the kidney of diabetic male Sprague Dawley rats, 3 weeks after STZ injection [14]. Treatment with NaHS during the 3rd week abolished the decrease in mRNA levels of the AT_1 receptor in STZ-induced diabetic rats, but did not alter the AT_1 receptor expression in nondiabetic rats [14].

A study of our group in a model of arterial hypertension induced by the infusion of Ang II in male Sprague Dawley rats showed that Ang II increased H_2O_2 production and the protein expression of Nox4 and AT_1 receptor in the renal medulla, but not in the renal cortex [17]. Noteworthy, treatment of Ang II-infused rats with PEG-catalase from day 7 to day 14 significantly reduced H_2O_2 production and the expression of Nox4 and AT_1 receptors in the renal medulla, thus suggesting that Ang II-derived H_2O_2 in the renal medulla stimulates the expression of Nox4 and AT_1 receptors[17].

The upregulation of intrarenal AT_1 receptor has also been evidenced in models of renal disease. Female WKY rats subjected to UNX and treated with bovine serum albumin for 4 weeks had increased $O_2^{\bullet-}$ generation and upregulation of AT_1 receptor mRNA and protein in the renal cortex [82]. Treatment of protein-overload UNX rats with apocynin for 3 weeks reduced renal cortical $O_2^{\bullet-}$ production and AT_1 receptor mRNA and protein levels [82]. Similar effects were also observed in male Sprague Dawley rats subjected to UNX and treated with AOPP-modified albumin [83]. These UNX rats treated with AOPP-modified albumin also showed increased renal cortical $O_2^{\bullet-}$ generation, as well as an augmented expression of the mRNA and protein of AT_1 receptor [83]. As previously observed in protein-overload UNX rats, treatment with apocynin also reduced the production of $O_2^{\bullet-}$ and the mRNA and protein levels of the AT_1 receptor in the renal cortex of AOPP-albumin-challenged rats [83]. The effects observed for the *in vivo* treatment with high levels of albumin or with AOPP-modified albumin on ROS production and AT_1 receptor expression were also reproduced in *in vitro* assays using cultured RPTCs (NRK52E) [82, 83].

In the heart, the mechanisms linking oxidative stress to altered AT_1 expression were investigated in fibroblasts prepared from young adult (2–3 months old) male Sprague Dawley rats

[22]. Treatment of cardiac fibroblasts with H_2O_2 caused a sixfold increase in AT_1 receptor mRNA levels in 3 hours, which were reduced to twofold at the end of 12 hours. AT_1 receptor protein expression was also significantly increased with maximum values reached at 6 and 12 hours of H_2O_2 treatment [22]. The preincubation of cardiac fibroblasts with the NADPH inhibitors DPI or VAS2870 abolished the H_2O_2-induced increase in AT_1 receptor mRNA and protein levels. Treatment with DPI also inhibited the H_2O_2-induced increase in intracellular ROS in cardiac fibroblasts [22]. Further experiments also showed that H_2O_2 induced the activation of NF-κB and activator protein 1 (AP-1) in cardiac fibroblasts and that preincubation of these cells for 60 min with the NF-κB inhibitor BAY-11-7085 or with the AP-1 inhibitor SR11302 prior to H_2O_2 treatment attenuated the AT_1 mRNA and protein expression. These data demonstrate that the H_2O_2-induced increase of AT_1 receptor mRNA and protein expression in cardiac fibroblasts involves the activation of NF-κB and AP-1 [22]. In subsequent experiments, H_2O_2 was also shown to increase by threefold the local secretion of Ang II. In addition, treatment with Ang II augmented the AT_1 receptor mRNA and protein expression in cardiac fibroblasts and these effects were significantly reduced by pretreatment with VAS2870. Therefore, it was concluded that Ang II increases the AT_1 receptor mRNA and protein expression in cardiac fibroblasts via NADPH oxidase-dependent ROS [22]. Moreover, H_2O_2 treatment significantly increased collagen mRNA and protein expression in these cells and the AT_1 receptor antagonist candesartan decreased these effects [22]. Overall, these findings suggest the existence of a positive feedback loop involving the reciprocal regulation of ROS, Ang II and the AT_1 receptor, which sustains the Ang II pathological signaling in the heart [22].

There have been contradictory reports regarding the effects of ROS on the AT_1 receptor regulation in the vasculature [135, 144]. In a study aimed at characterizing the second messengers used by Ang II in the regulation of AT_1 receptor gene expression, Nickenig et al. showed that treatment with Ang II caused a significant release of ROS in VSMCs and a downregulation in AT_1 receptor mRNA and density in cultured VSMCs isolated from the thoracic aorta of 6–10-week old female WKY rats. Coincubation with DPI significantly inhibited the Ang II-induced ROS release and the downregulation in AT_1 receptor mRNA [135]. VSMCs were also incubated with a mixture of H_2O_2 and ferric nitrilotriacetate or with xanthine oxidase plus purine in order to evaluate if ROS have direct effects on AT_1 receptor expression. Both H_2O_2 and xanthine oxidase induced a dose-dependent downregulation in AT_1 receptor mRNA. H_2O_2 also decreased the AT_1 receptor protein expression [135]. Further experiments demonstrated that although H_2O_2 did not alter the AT_1 receptor mRNA transcription rate it caused a marked decrease in the AT_1 receptor mRNA half-life, thus suggesting that ROS destabilize the AT_1 receptor mRNA [135]. These findings identify ROS as possible mediators of Ang II-induced downregulation of the AT_1 receptor and suggest that ROS-mediated negative feedback regulation of AT_1 receptor is a cellular self-protecting mechanism that limits the potential pathological effects of the exposure of VSMCs to high concentrations of ROS generated in response to prolonged AT_1 receptor activation [135]. In contrast to these results, Bhatt et al. demonstrated that augmented vascular oxidative stress caused an upregulation of the AT_1 receptor in human aortic smooth muscle cells and in arteries from 11 to 12 weeks old SHR [144]. Treatment of these cells for 24 hours with BSO or with H_2O_2 for 3 hours failed to induce a significant increase in AT_1 receptor mRNA. However, the combination of these oxidants elicited a twofold increase in the AT_1 receptor mRNA, as well as an increase in

oxidative stress. These effects were prevented by the simultaneous treatment with catalase. Moreover, in the presence of p65 siRNA, the oxidant treatment did not increase the AT_1 receptor mRNA [144]. In SHR, but not in WKY rats, vascular oxidative stress was also increased, as evidenced by augmented H_2O_2 levels and was associated with increased vascular protein expression of NF-κB and AT_1 receptor and enhanced vasoconstriction in response to Ang II. Treatment with the antioxidant and NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) for 6–7 weeks reduced blood pressure, vascular H_2O_2 levels, p65 overexpression, AT_1 receptor expression and Ang II-induced vasoconstriction [144]. Together, these results indicate that under conditions of enhanced oxidative stress there is an upregulation of vascular AT_1 receptor that possibly involves ROS-induced NF-κB activation. Furthermore, the blood pressure lowering the effect of PDTC might have resulted from the normalization of vascular AT_1 receptor expression and prevention of exaggerated vasoconstriction to Ang II [144]. In addition to the stimulation of vascular AT_1 receptor expression, ROS may also enhance the vascular response to Ang II by increasing the functional availability of AT_1 receptors [134]. Under physiological conditions, AT_1 receptors are rapidly desensitized and internalized on stimulation by Ang II, thus avoiding an excessive responsiveness to Ang II [91, 134]. However, in pathological conditions such as arterial hypertension this mechanism might be compromised thus resulting in sustained activation of AT_1 receptors [134]. Bagi et al. tested the hypothesis that the acute exposure of resistance arteries to high intraluminal pressure increases the constriction to Ang II via a ROS-mediated improvement in the functional availability of AT_1 receptors [134]. In this study, performed in gracilis arterioles isolated from male Wistar rats, they observed that the transient exposure of the vessels to high intraluminal pressure (160 mmHg) significantly increased the constrictions to the second application of Ang II. This response was reduced by the AT_1 receptor antagonist telmisartan but not by the selective AT_2 receptor blocker PD123,319. In addition, preincubation of the arterioles with tiron or with PEG-catalase prevented the high intraluminal pressure-induced increase of arteriolar constrictions to the second application of Ang II [134]. Furthermore, the transient exposure to H_2O_2 resulted in augmented vessel constriction in response to the second application of Ang II. Overall, these findings indicate that ROS, especially H_2O_2, contribute to the high pressure-induced increase of the vasoconstriction to Ang II. This pathological feedforward mechanism may therefore lead to increased vascular resistance and amplify the hypertensive state [134].

The effects of oxidative stress on AT_1 receptor expression were also studied in macrophages, since Ang II is a proatherogenic molecule and both oxidative stress and AT_1 receptor expression are increased in hypercholesterolaemia [145–147]. In mouse peritoneal macrophages (MPMs) harvested from the E^0 mice, an animal model of severe hypercholesterolemia and atherosclerosis caused by apolipoprotein E deficiency, there was an age-dependent increase in lipid peroxide content accompanied by an age-dependent increase in the AT_1 receptor mRNA and protein expression [146]. MPMs obtained from 3.5 months old E^0 mice treated for 6 weeks with the potent antioxidant vitamin E had lower lipid peroxides concentration and reduced AT_1 receptor mRNA expression, compared to MPMs harvested from untreated E^0 mice [146]. To further demonstrate the role of oxidative stress in the regulation of macrophage AT_1 receptor, the GSH content was manipulated by the supplementation for 5 weeks with BSO or with L-2-oxothiazolidine-4-carboxylic acid (OTC), a precursor of GSH synthesis. It was observed that the reduction in macrophage GSH content was associated with increased AT_1

receptor mRNA expression, whereas the elevation of macrophage GSH levels caused a lower expression of AT_1 receptor mRNA. Similar effects of BSO and OTC on the AT_1 receptor mRNA expression were shown in MPMs obtained from control BALB/c mice [146]. Moreover, oxidized LDL, but not native LDL, caused a significant dose-dependent increase in AT_1 receptor mRNA and protein levels in MPMs from BALB/c mice [146]. These results suggest that oxidative stress enhances the proatherogenic effects of Ang II by inducing the overexpression of AT_1 receptors in arterial macrophages [146].

The regulation of AT_1 receptor by oxidative stress was also investigated in the central nervous system of male New Zealand white rabbits with chronic heart failure (CHF) [148]. It is well known that activation of the RAAS and of the sympathetic nervous system in CHF critically contributes to the development and progression of this pathological syndrome [148–151]. Previous studies have shown that CHF animals exhibit an upregulation of central AT_1 receptor and that the stimulation of sympathetic outflow by central Ang II treatment is mediated by oxidative stress via stimulation of NADPH oxidase-derived ROS production [148, 152–154]. Furthermore, NADPH oxidase-derived ROS in the rostral ventrolateral medulla (RVLM) are involved in the Ang II-induced pressor responses [155]. Therefore, Liu et al. evaluated the relationship between oxidative stress, antioxidant treatment and AT_1 receptor regulation in a neuronal cell line and in the RVLM of CHF rabbits. They observed that treatment of CATH.a cells with Ang II markedly increased the AT_1 receptor mRNA expression, NADPH oxidase activity and $O_2^{\bullet-}$ generation [148]. These effects on the AT_1 receptor expression and oxidative stress were inhibited by the AT_1 receptor antagonist losartan, apocynin and tempol, thus suggesting that there is a positive feedback mechanism whereby Ang II upregulates the AT_1 receptor expression via increased ROS production [148]. In the RVLM of CHF rabbits that received an intracerebroventricular infusion of tempol for 7 days AT_1 receptor mRNA and protein expression was significantly reduced when compared to vehicle-infused CHF rabbits. Furthermore, they also verified that the RVLM AP-1 binding activity that was previously shown to be increased in CHF rabbits, compared to sham rabbits, was decreased by the intracerebroventricular administration of tempol to CHF rabbits [148]. Collectively, these findings indicate that ROS play a major role in the central upregulation of AT_1 receptor expression in CHF.

Currently, there is a lack of studies regarding the regulation of AT_2 and MAS receptors by ROS. To our knowledge, only one study explored the impact of oxidative stress on AT_2 and MAS receptor expression. The evaluation of mRNA and protein expression of RAAS components in the renal cortex of 10-week-old male obese Zucker rats revealed that there was an increase in the AT_1 receptor accompanied by augmented AT_2 receptor expression and lower expression of MAS receptor, compared to lean Zucker rats [84]. In addition, obese rats also exhibited a greater diuretic and natriuretic response to the AT_2 receptor agonist CGP-42112A and a lower Ang (1–7)-mediated natriuresis than lean rats [84]. Treatment with tempol for 4 weeks further increased AT_2 receptor expression, as well as the AT_2 receptor-mediated diuretic and natriuretic responses in obese, but not lean rats. It also decreased AT_1 receptor expression and increased MAS receptor expression and the diuretic effect of Ang (1–7) in obese rats but not in lean Zucker rats [84]. In agreement with the *in vivo* data, cultured RPTCs obtained from 14-week-old obese Zucker rats showed higher protein expressions of AT_1 and AT_2 receptors, but decreased protein expression of MAS receptor when compared with cells from lean Zucker rats [84]. *In vitro* treatment with tempol for 24 hours reduced AT_1 receptor expression, increased the expression

of MAS receptor and further increased the expression of AT_2 receptor expression [84]. These results suggest that in obesity the supplementation with antioxidants may correct the balance between natriuretic and antinatriuretic components of the renal RAAS [84].

Figures 7 and **8** summarize the effects of ROS on Ang receptors.

AT$_1$ receptor

KIDNEY:

❑ $O_2^{\bullet-}$ ↑ AT_1 receptor mRNA and protein expression and ligand binding in Sprague Dawley rats treated with BSO (inhibitor of GSH synthesis)

❑ $O_2^{\bullet-}$ ↑ AT_1 receptor mRNA and protein expression in the renal cortex of obese Zucker rats, but not in lean Zucker rats

❑ $O_2^{\bullet-}$ ↑ AT_1 receptor-mediated effects on water and sodium excretion in obese Zucker rats (but not in lean Zucker rats) and in aged rats (21 months old) (but not in adult rats) (3 months old)

❑ $O_2^{\bullet-}$ ↑ AT_1 receptor protein expression in the renal cortex of SHR fed a HF diet, but not in SHR fed a NF diet or in WKY rats fed a HF or NF diet.

❑ $O_2^{\bullet-}$ ↑ AT_1 receptor protein expression in STZ-induced diabetic Sprague Dawley rats

❑ H_2O_2 ↑ AT_1 receptor protein expression in the renal medulla (but not in renal cortex) of Ang II-hypertensive rats.

❑ NADPH oxidase-derived ROS ↑ AT_1 receptor mRNA and protein expression in the renal cortex of UNX rats (model of hypertension and renal injury) treated with albumin or with AOPP-modified albumin

KIDNEY – proximal tubular cells:

❑ $O_2^{\bullet-}$ ↑ AT_1 receptor protein expression (in cells isolated from obese Zucker rats but not in those from lean Zucker rats)

❑ NADPH oxidase-derived ROS ↑ AT_1 receptor protein expression in cells exposed to albumin or to AOPP-modified albumin

KIDNEY – mesangial cells:

❑ NADPH oxidase-derived ROS ↑ AT_1 receptor mRNA expression in cells exposed to HG medium; no effect in NG conditions

MACROPHAGES:

❑ ROS ↑ AT_1 receptor mRNA expression in peritoneal macrophages from the E^0 mice (model of severe hypercholesterolemia /atherosclerosis) and from controls (Balb/C mice)

HEART – fibroblasts:

❑ H_2O_2 ↑ AT_1 receptor mRNA and protein expression

❑ NADPH oxidase-derived ROS ↑ AT_1 receptor mRNA and protein expression in response to Ang II

ARTERIES:

❑ ROS ↑ AT_1 receptor protein expression and AT_1 function (Ang II-induced vasoconstriction)

❑ ROS (especially H_2O_2) ↑ the functional availability of vascular AT_1 receptors in response to high intraluminal pressure

ARTERIES – Smooth muscle cells:

❑ NADPH oxidase-derived ROS ↓ AT_1 receptor mRNA expression in response to Ang II in VSMCs

❑ H_2O_2 and xanthine oxidase-derived ROS ↓ AT_1 receptor mRNA expression in VSMCs

❑ H_2O_2 ↓ AT_1 receptor mRNA half-life in VSMCs

❑ H_2O_2 plus BSO (a GSH synthesis inhibitor) ↑ AT_1 receptor mRNA expression in HASMCs

CENTRAL NERVOUS SYSTEM:

❑ NADPH oxidase-derived ROS ↑ AT_1 receptor mRNA expression in response to Ang II in neuronal (CATH.a) cells

❑ $O_2^{\bullet-}$ ↑ AT_1 receptor mRNA and protein expression in the RVLM of CHF rabbits
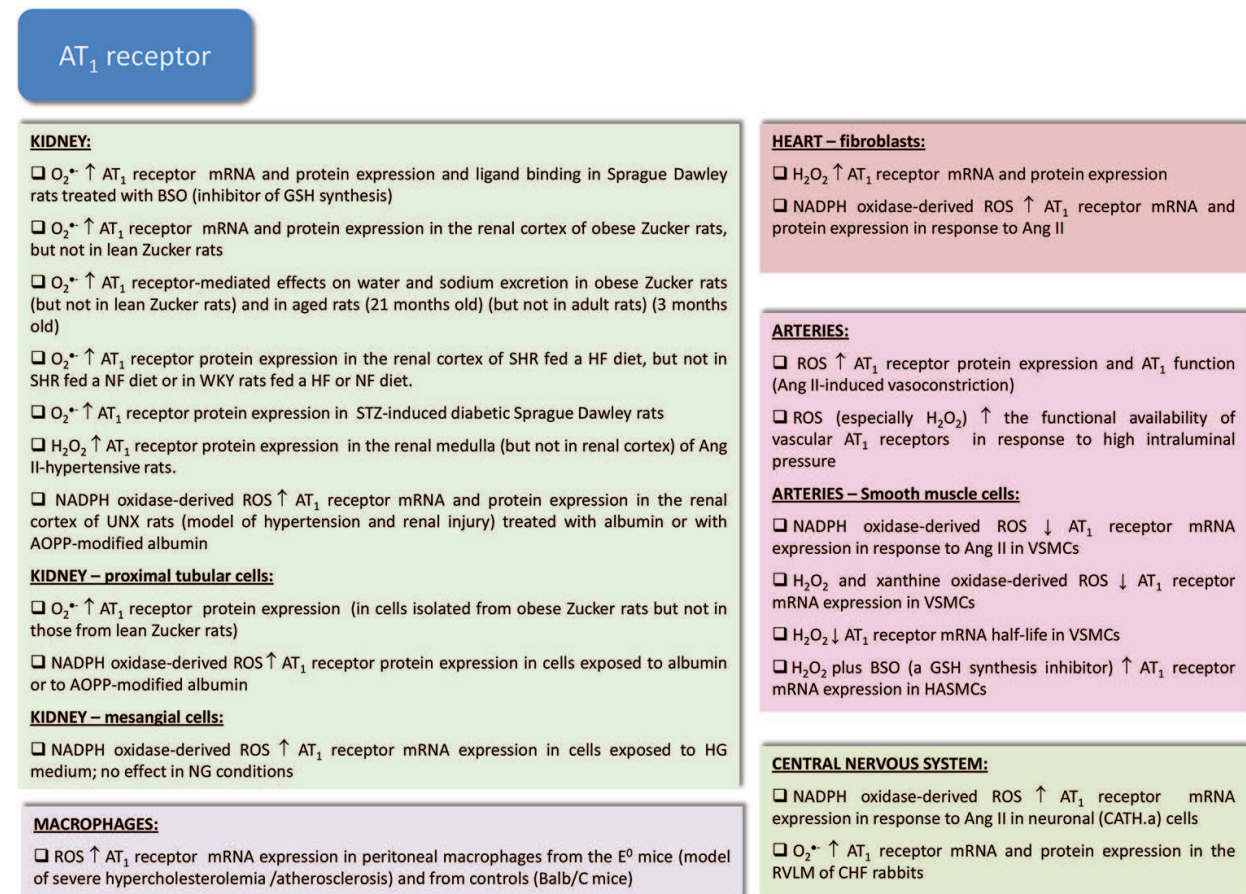
Figure 7. Regulation of AT_1 receptor by ROS. Ang II, angiotensin II; AOPP, advanced oxidation protein products; BSO, L-buthionine sulfoximine; CHF, chronic heart failure; GSH, glutathione (reduced form) HASMCs, human aortic smooth muscle cells; HF, high-fat; HG, high-glucose; NF, normal-fat; NG, normal-glucose; ROS, reactive oxygen species; RVLM, rostral ventrolateral medulla; SHR, Spontaneously Hypertensive Rat; STZ, streptozotocin; UNX, uninephrectomized; VSMCs, vascular smooth muscle cells; WKY, Wistar Kyoto.
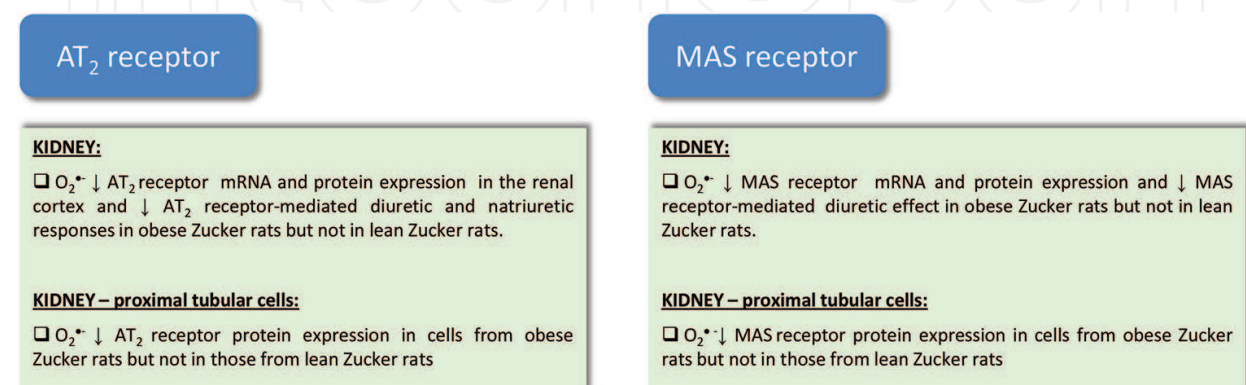
AT$_2$ receptor

KIDNEY:

❑ $O_2^{\bullet-}$ ↓ AT_2 receptor mRNA and protein expression in the renal cortex and ↓ AT_2 receptor-mediated diuretic and natriuretic responses in obese Zucker rats but not in lean Zucker rats.

KIDNEY – proximal tubular cells:

❑ $O_2^{\bullet-}$ ↓ AT_2 receptor protein expression in cells from obese Zucker rats but not in those from lean Zucker rats

MAS receptor

KIDNEY:

❑ $O_2^{\bullet-}$ ↓ MAS receptor mRNA and protein expression and ↓ MAS receptor-mediated diuretic effect in obese Zucker rats but not in lean Zucker rats.

KIDNEY – proximal tubular cells:

❑ $O_2^{\bullet-}$ ↓ MAS receptor protein expression in cells from obese Zucker rats but not in those from lean Zucker rats

Figure 8. Regulation of AT_2 receptor and MAS receptor by ROS.

3. Conclusions

A plethora of experimental evidence indicates that ROS are important upstream regulators of the expression, secretion and/or activity of RAAS components. The majority of the referred studies suggests that under conditions of increased ROS availability there is an enhanced RAAS activation that is attenuated or abolished by treatment with antioxidants or inhibitors of ROS production. Nevertheless, there are also some reports of negative regulation of RAAS constituents by oxidant species that might serve as physiological protective mechanisms limiting the overactivation of this system and consequent deleterious effects on cell and organ functions. Importantly, in experimental pathological conditions associated with increased oxidative stress, such as arterial hypertension, obesity, diabetes, heart failure and renal disease, ROS have been shown to promote RAAS upregulation, thereby inducing a positive feedback loop that aggravates the cardiometabolic and/or renal injury. Currently, there is a lack of clinical studies evaluating the impact of the manipulation of ROS levels by antioxidants or inhibitors of ROS production on the expression, secretion and activity of RAAS components. The elucidation of the role of ROS in the regulation of RAAS in human physiological and pathological conditions, as well as the development of dual antioxidant-cardiovascular acting drugs and comparison of their clinical efficacy over currently used agents, would be important to improve the therapeutic strategies for many pathologies for which the blockade of RAAS appears to be insufficient to prevent disease-associated morbidity and mortality due to the existence of escape mechanisms.

Acknowledgements

Abbreviations

AP-1	activator protein 1
AOPPs	advanced oxidation protein products
CYP11B2	aldosterone synthase
AT$_1$	Ang II type 1
AT$_2$	Ang II type 2
Ang (1–7)	angiotensin (1–7)
Ang (1–9)	angiotensin (1–9)
Ang III	angiotensin 2–8
Ang IV	angiotensin 3–8
ACE	angiotensin-converting enzyme
Ang I	angiotensin I
Ang II	angiotensin II
ACE2	angiotensin-converting enzyme 2
AGT	angiotensinogen
CHF	chronic heart failure
CD36	cluster of differentiation 36

cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
COX-2	cyclooxygenase-2
DPI	diphenylene iodonium
DJ-1$^{-/-}$	DJ-1-knockout mice
eNOS	endothelial nitric oxide synthase
ET-1	endothelin 1
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FBN	Fischer 344 Brown Norway F1
GSH	glutathione
GPCR	G-protein-coupled receptor
GTP	guanosine triphosphate
HO-1	heme oxygenase-1
HDL	high-density lipoprotein
H_2O_2	hydrogen peroxide
$^{\bullet}OH$	hydroxyl radical
PVN	hypothalamic paraventricular nucleus
IRPTCs	immortalized renal proximal tubule cells
JNK	Jun Kinase
JG	juxtaglomerular
OTC	L-2-oxothiazolidine-4-carboxylic acid
BSO	L-buthionine sulfoximine
LpA	lipoprotein A
LDL	low-density lipoprotein
MR	mineralorticoid receptor
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemoattractant protein 1
MPMs	mouse peritoneal macrophages
NAC	N-acetylcysteine
Nox	NADPH oxidase isoform
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
Nrf2	Nuclear factor erythroid 2-related factor 2
NF-κB	nuclear factor kappa B
PMA	phorbol 12-myristate 13-acetate
PLC	phospholipase C
PCR	polymerase chain reaction
PA	primary aldosteronism
PRR	(pro)renin receptor (PRR)
PKA	protein kinase A
PKC	protein kinase C
PDTC	pyrrolidine dithiocarbamate
ROS	reactive oxygen species

RPTCs	renal proximal tubule cells
RAAS	renin-angiotensin-aldosterone system
RVLM	rostral ventrolateral medulla
SHR	spontaneously hypertensive rats
STZ	streptozotocin
SOD	superoxide dismutase
$O_2^{\bullet-}$	superoxide radical
TBARS	thiobarbituric reactive substances
TGFβ	transforming growth factor β
TNFα	tumor necrosis factor α
NOS1	type 1 nitric oxide synthase
UNX	unilateral nephrectomy
VSMCs	vascular smooth muscle cells
WB	Western Blot
WT	wild-type
WKY	Wistar-Kyoto
XOD	xanthine/xanthine oxidase
ZDF	Zucker Diabetic Fatty

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