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出版情報：Nitric Oxide. 25 (2), pp.112-117, 2011-08-01. Elsevier
バージョン：

Review Article

Regulation of Angiotensin II receptor signaling by cysteine modification of NF-κB

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Abstract

Angiotensin II (Ang II) is a major vasoactive peptide of the renin-angiotensin system. Ang II is originally found as one of potent vasoconstrictors, but is now attracted attention as an essential mediator of many cardiovascular problems, including endothelial dysfunction, arrhythmia and structural remodeling of cardiovascular systems. Most of the known pathophysiological effects of Ang II are mediated through Ang type1 receptors (AT_1Rs), and the up-regulation of AT_1Rs is one of important causes by which Ang II can contribute to cardiovascular diseases. A growing body of evidence has suggested that reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles in the regulation of AT_1R signaling. In cardiac fibroblasts, stimulation with cytokines or bacterial toxins induces AT_1R up-regulation through NADPH oxidase-dependent ROS production. In contrast, nitric oxide (NO) decreases AT_1R density through cysteine modification (S-nitrosylation) of a transcriptional factor, nuclear factor κB (NF-κB). The difference between the effects of ROS and NO on AT_1R expression may be caused by the difference between intracellular location of ROS signaling and that of NO signaling, as the agonist-induced S-nitrosylation of NF-κB requires a local interaction between NO synthase (NOS) and NF-κB in the perinuclear region. Thus, the spatial and temporal regulation of cysteine modification by ROS or RNS may underlie the resultant changes of AT_1R signaling induced by agonist stimulation.

Keywords: Angiotensin, AT_1 receptor, heart, reactive oxygen species, nuclear factor-κB, S-nitrosylation

1. Ang II signaling in the heart

The renin-angiotensin system (RAS) plays an important role in maintaining blood pressure homeostasis and salt balance in mammals [1, 2]. Ang II, a key regulator of RAS and exerts biological functions [3, 4], is generated to the following pathway. The protease renin first cleaves angiotensinogen to generate Ang I, which is composed of 10 amino acid polypeptide (Ang (1-10)). Next, the Ang converting enzyme (ACE) cleaves Ang I to generate Ang II (Ang (1-8)). Although there exists alternative Ang II-generating enzymes, such as cathepsins and chymase [5, 6], ACE is believed to be the sole enzyme in the regulation of Ang II production in the RAS [1, 2]. Ang II is then degraded to the heptapeptide Ang (1-7) by ACE2 [7]. Several reports have shown a counter-regulatory role for Ang (1-7) by opposing many AT_1R-mediated actions [8, 9]. The Ang (1-7) is further degraded by aminopeptidase to generate Ang III (Ang (2-8)) and Ang IV (Ang (3-8)). These Ang-derived metabolites exert biological activities through binding to G protein-coupled receptors (GPCRs) including AT_1R, AT_2R, and Mas [10, 11]. Among the Ang-derived metabolites, Ang II is a key regulator of cardiovascular functions, and most of biological effects of Ang II are mediated via AT_1R [3, 4, 10-12].

AT_1R-mediated signaling is divided into two groups: G protein-dependent and G protein-independent signaling pathways [13]. AT_1Rs are known to couple with three G protein subfamilies; G_q, G_i, G_{12} [13, 14]. Among them, G_q and G_{12} family proteins-mediated signaling pathways participate in structural remodeling of the heart (Figure 1). The G_q family protein induces activation of phospholipase C (PLC), which in turn induces increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) through production inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) by hydrolyzing

phosphatidylinositol 4,5-bisphosphate (PIP_2). Sustained increase in $[Ca^{2+}]_i$ in cardiomyocytes induces activation of calmodulin (CaM)-dependent protein phosphatase 2B, calcineurin, which leads to increase in expression of hypertrophic genes, such as atrial natriuretic peptide (ANP), β-myosin heavy chain (β-MHC), and α-skeletal muscle actin (α-SKA), through activation of nuclear factor of activated T cells (NFAT) [15]. We have recently reported that DAG-activated transient receptor potential canonical (TRPC) channels (TRPC3 and TRPC6) mediate Ang II-induced Ca^{2+} influx through voltage-dependent Ca^{2+} channel (VDCC) and cardiac hypertrophy *in vivo* and *in vitro* [16, 17]. In addition, activation of extracellular signal-regulated kinase (ERK) signaling pathway also participates in G_q-mediated cardiac hypertrophy. Lorenz *et al*. [18] has reported that Gβγ released from $G\alpha_q$ induces autophosphorylation of ERK1/2 at Thr^{188} residue and subsequent nuclear translocation of ERK1/2, resulting in inducing expression of hypertrophic genes. Ang II also induces ROS production through NADPH oxidase activation [19, 20]. Interestingly, Ang II-induced ROS production through Nox2 activation attenuates basal endothelial NOS (eNOS) activity through proline-rich tyrosine kinase (Pyk2)-dependent phosphorylation of eNOS at Tyr657 in endothelial cells [21], suggesting the mechanism of endothelial dysfunction observed in cardiovascular diseases associated with increased activity of the RAS. Using rat neonatal cardiomyocytes, we found that G_{12} family proteins mediate Ang II-induced ROS production and activation of c-Jun NH_2-terminal kinase (JNK) and p38 MAPK [22, 23]. The activation of apoptosis signal-regulating kinase (ASK) and protein tyrosine kinase (PTK) by cysteine oxidation of thioredoxin and protein tyrosine phosphatase 1B may participate in ROS-mediated activation of JNK and p38MAPK [3, 4]. These results suggest that ROS function as a second messenger of $G_{12/13}$ signaling in

cardiomyocytes. Furthermore, the inhibition of $G\alpha_{12/13}$ signaling in cardiomyocytes attenuates pressure overload-induced collagen deposition of the heart (fibrosis), using transgenic mice with cardiomyocytes-specific overexpression of regulator of G protein signaling (RGS) domain of p115 Rho guanine nucleotide exchange factor (p115RhoGEF) [24]. These results suggest that $G\alpha_{12/13}$-mediated ROS production contributes to cardiac fibrosis induced by pressure overload. As cardiac fibrosis is one of major causes of left ventricular diastolic dysfunction, and the inhibition of $G\alpha_{12/13}$-mediated ROS signaling attenuated diastolic dysfunction, $G\alpha_{12/13}$-mediated ROS signaling may be a novel therapeutic target for the treatment of heart failure.

2. Redox regulation of AT_1R proteins

Expression cloning from bovine adrenal and rat smooth muscle cells has revealed that both AT_1Rs are typical seven transmembrane domain proteins, composed of 359 amino acids and a molecular mass of about 41 kDa [25]. As the AT_1R possesses three N-glycosylation sites and four cysteine residues in the extracellular regions, the native glycosylated AT_1R has a molecular mass of about 65 kDa [26]. Although the rodent AT_1Rs exist as two distinct subtypes, $AT_{1A}R$ and $AT_{1B}R$, that are 95% identical in their amino acid sequences, these structural features are present in several other mammalian and nonmammalian AT_1Rs [10, 27]. The AT_1Rs are not only activated by Ang-derived metabolites, but also activated by mechanical stretch [28, 29]. The disulfide bridges between the N-terminal region and third extracellular loop, and between the first and second extracellular loops are formed to maintain the conformation of the AT_1R protein [30]. As the disulfide bridge between the N-terminal region and third extracellular loop is not present in AT_2R, the AT_2R is resistant to inactivation by reducing agents.

Furthermore, Zhang *et al*. [31] has recently reported that nitro-oleic acid specifically binds to the AT_1R, reduces G_q protein coupling, and inhibits inositol-1,4,5-trisphosphate production and Ca^{2+} mobilization, without inhibiting Ang II binding to the receptor. Thus, the AT_1R-mediated signaling is negatively regulated by the oxidation or reduction of the AT_1Rs.

3. Regulation of AT_1R expression level

The gene expression of AT_1R is regulated by various transcriptional factors, such as Sp1, Sp3, myocyte enhancing factor (MEF)-2, peroxisome proliferator-activated receptor (PPAR)-γ, and NF-κB [32-36]. It has been reported that Sp1 and Sp3 are predominantly responsible for regulating the basal expression of the human AT_1R gene [33, 37]. In contrast, MEF-2 heterodimers and Sp1 are required for basal expression of the rat $AT_{1A}R$ gene [34]. PPAR-γ, an anti-inflammatory transcriptional factor, and Sp1 directly interact with each other on a CG-box-related sequence within the -58/-34 bp region of the rat $AT_{1A}R$ promoter, which leads to suppression of $AT_{1A}R$ transcription by inhibiting Sp1 binding activity [32]. On the other hand, NF-κB, an inflammatory transcriptional factor, has been reported to participate in up-regulation of rat $AT_{1A}R$ density induced by proinflammatory cytokines [36, 38, 39]. Cowling *et al*. [36] has reported that NF-κB binds 2 *cis*-reponse elements located at -365/-355 bp and -2540/-2530 bp and induce transactivation of a minimal promoter. They have indicated that proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), increase the expression level of $AT_{1A}R$ (Figure 2). Transcription of the rat $AT_{1A}R$ is also enhanced by Toll-like receptor 4 (TLR4) ligands, such as lipopolysaccharide (LPS) and oxidized low-density lipoprotein (LDL), both of

which strongly increase NF-κB activity. In contrast, NO, growth factors, statins and 15-deoxy-δ-PGJ_2 decrease AT_1R density [32, 40-43]. This mechanism may be explained by the decrease in DNA binding affinity of transcriptional factors through oxidative modification [43]. In addition, Ang II, cAMP stimulating agents and estrogens increase AT_1R mRNA decay rates, leading to decrease in AT_1R density (Figure 2). The AT_1R mRNA stability is regulated by calreticulin. The phosphorylated calreticulin binds to AUUUUA sequence localized in 3'-UTR region of AT_1R mRNA, leading to increase in AT_1R mRNA stability [44]. Thus, both transcriptional and posttranscriptional mechanisms are involved in the regulation of AT_1R expression.

4. ROS-mediated up-regulation of AT_1Rs

Ichiki *et al*. [45] have previously reported that exogenous exposure of hydrogen peroxide (H_2O_2) decreases AT_1R density through AT_1R mRNA destabilization. As the Ang II-induced decrease in AT_1R mRNA stability was diminished by the treatment with diphenyleneiodonium (DPI), an NADPH oxidase inhibitor, they suggest that endogenous ROS derived from NADPH oxidase negatively regulate AT_1R mRNA stability. In contrast, treatment with IL-1β and TNF-α induces NADPH oxidase-dependent ROS production and increases AT_1R density [38, 39, 46]. Therefore, the role of endogenous ROS in the regulation of AT_1R expression may depend on the location and timing of ROS production induced by agonist stimulation.

We have recently found that endogenous ROS increase AT_1R density in rat cardiac fibroblasts [47]. *Pertussis* toxin (PTX), a major virulence factor of Gram-negative bacillus *Bordetella* pertussis which causes whooping cough, is well established as a

pharmacological tool for a specific inhibitor of receptor-$G_{i/o}$ protein coupling. PTX is composed of A-protomer and B-oligomer, and A-protomer exerts ADP-ribosyltransferase activity on the α-subunit of heterotrimeric G_i proteins ($G\alpha_i$), leading to inhibition of receptor-G protein coupling, whereas B-oligomer of PTX recognizes and binds carbohydrate-containing receptors that deriver A-protomer into the cytosol. As the treatment of rat cardiac fibroblasts with PTX induces up-regulation of AT_1Rs, indicating that PTX increases AT_1R density independently of ADP ribosylation. We also found that PTX induces TLR4 stimulation, which leads to activation of a small GTP-binding protein, Rac, through Syk tyrosine kinase (Figure 3). Activation of Rac subsequently induces NF-κB activation through IκBα phosphorylation and degradation. Activation of NF-κB then increases expression of IL-1β, which in turn activates Rac through IL-1β receptor stimulation. Thus, IL-1β-dependent amplification of ROS-mediated NF-κB signaling is required for the PTX-induced AT_1R up-regulation. It is still unclear how endogenous ROS induce phosphorylation and degradation of IκBα, but recent studies have suggested that stress-induced Ca^{2+} mobilization induces nuclear accumulation of IκBα, leading to IκBα degradation without N-terminal phosphorylation of IκBα [48, 49]. As the stimulation of TLR4 induces ROS production independently of MyD88 signaling pathways has also been reported in macrophages [50], ROS-mediated signaling induced by TLR4 stimulation may play a critical role in TLR4-induced inflammatory responses (i.e., AT_1R up-regulation) in mammalian cells.

5. Suppression of AT_1R signaling by S-nitrosylation

It has been reported that NO and nitro-unsaturated fatty acids decrease Ang II

signaling in the heart [31, 43]. We have recently found that long-term treatment of rat neonatal cardiac fibroblasts with adenosine 5'-triphosphate (ATP) decreases AT_1R density. This phenomenon is so-called 'heterologous down-regulation', which indicates that stimulation of one GPCR reduces expression levels of different GPCR [51]. Cross-talk between different GPCR signaling pathways may serve to fine-tune cell signaling [52], but the molecular mechanism(s) underlying heterologous down-regulation is largely unknown. The ATP-induced AT_1R down-regulation was canceled by the treatment with cyclosporine A or siRNAs for $P2Y_2R$, indicating that $P2Y_2R$-stimulated sustained increase in intracellular Ca^{2+} concentration and subsequent activation of calcineurin-NFAT signaling participate in ATP-induced AT_1R down-regulation. Interestingly, the ATP-induced suppression of AT_1R signaling was completely diminished by 1400W, a selective inhibitor of inducible NO synthase (iNOS). As iNOS promoter region contains some NFAT-binding sequences and activation of calcineurin-NFAT signaling pathway has been reported to induce expression of iNOS proteins in cardiomyocytes and *in vivo* mouse hearts [53]. In fact, ATP increased expression of iNOS proteins in a concentration-dependent manner, and ATP-induced iNOS expression was completely suppressed by cyclosporine A. The expression of constitutively active mutant of NFAT also decreased AT_1R density, which was canceled by 1400W. Thus, NFAT-dependent iNOS expression is required for ATP-induced suppression of AT_1R signaling. We focused on the conserved cysteine (Cys) residue in the Rel homology domain of NF-κB [54, 55]. The Cys wedged between acidic and basic amino acids is nucleophilic, acidic and redox active, and numerous reactions may occur on this Cys thiol side chain [56]. As the reactive Cys locates in the Rel homology domain that is involved in DNA binding or dimer

formation of p65, the NO-mediated Cys modification (S-nitrosylation) of p65 may decrease transcriptional activity of NF-κB [54, 55]. Using biotin-switch assay method as shown in Fig. 4A, we found that treatment of rat cardiac fibroblasts with ATP for 24 hours actually induced S-nitrosylation of p65 subunit at Cys38. The promoter region of iNOS does not only contain NF-κB binding sequences but also NFAT binding sequences (Fig. 4B). Treatment of cardiac fibroblasts with ATP induced iNOS expression, and the iNOS protein and p65 subunit were co-localized around the perinuclear regions (Fig. 4C). Immunoprecipitation study has revealed that ATP-induced iNOS forms a complex with p65. In contrast, treatment with IL-1β strongly increased expression of iNOS protein but did not induce the interaction of iNOS with p65, because IL-1β induces translocation of p65 from cytosol to the nucleus. We also found that flavin-binding domain of iNOS protein is a hot-spot in iNOS for the interaction with p65, and overexpression of iNOS fragment including flavin-binding domain completely suppressed the ATP-induced suppression of AT_1R signaling. Thus, the formation of signaling complex between iNOS and p65 around the perinuclear region may be essential for the ATP-induced AT_1R down-regulation.

6. Conclusion

Numerous studies have demonstrated that Cys modification by ROS and NO play important roles in agonist-induced signal transduction. Indeed, numerous proteins have been reported to be a target of NO [57]. We found that ROS and NO play important roles in the regulation of AT_1R gene expression. ROS increases AT_1R density through IκBα degradation-dependent activation of NF-κB, while NO decreases AT_1R density through direct S-nitrosylation of NF-κB p65 subunit. As the physical

interaction between p65 and iNOS proteins are required for agonist-induced S-nitrosylation of p65, the location and the timing of ROS or NO production induced by agonist stimulation may be critical for the resultant regulation of AT_1R signaling. Identification of the target protein that induces IκBα phosphorylation will be required for the understanding of the ROS-mediated signaling complex participating in AT_1R up-regulation. A growing body of evidence has suggested that ROS and NO work as second messengers in cellular signaling, and the emergence of Cys modification (thiol oxidation and S-nitrosylation) by ROS and RNS may presage a new era in cardiovascular biology. Unraveling the regulation of Ang II signaling by Cys modification will achieve new therapeutic targets with great potential to improve clinical outcomes.

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Figure legends

Figure 1. AT_1R signaling pathways in the heart. ASK; apoptosis signal-regulating kinase, PTK; protein tyrosine kinase, VDCC; voltage-dependent Ca^{2+} channel.

Figure 2. Regulation of AT_1R transcription and translation by various factors. PPAR; peroxisome proliferator-activated receptor, LDL; low-density lipoprotein.

Figure 3. Hypothetical mechanism of ROS-mediated AT_1R up-regulation induced by *Pertussis* toxin in rat cardiac fibroblasts.

Figure 4. Mechanism of AT_1R down-regulation induced by NO. (A) principle of biotin-switch assay. (B) Potential binding sites of transcriptional factors existed in iNOS promoter region. (C) Changes in localization of iNOS and p65 proteins induced by ATP and IL-1β. Cardiac fibroblasts were treated with ATP (100 μM) or IL-1β (1 ng/ml) for 24 hours. (D) Localization of p65 defines iNOS-dependent S-nitrosylation of p65 and resultant AT_1R down-regulation.

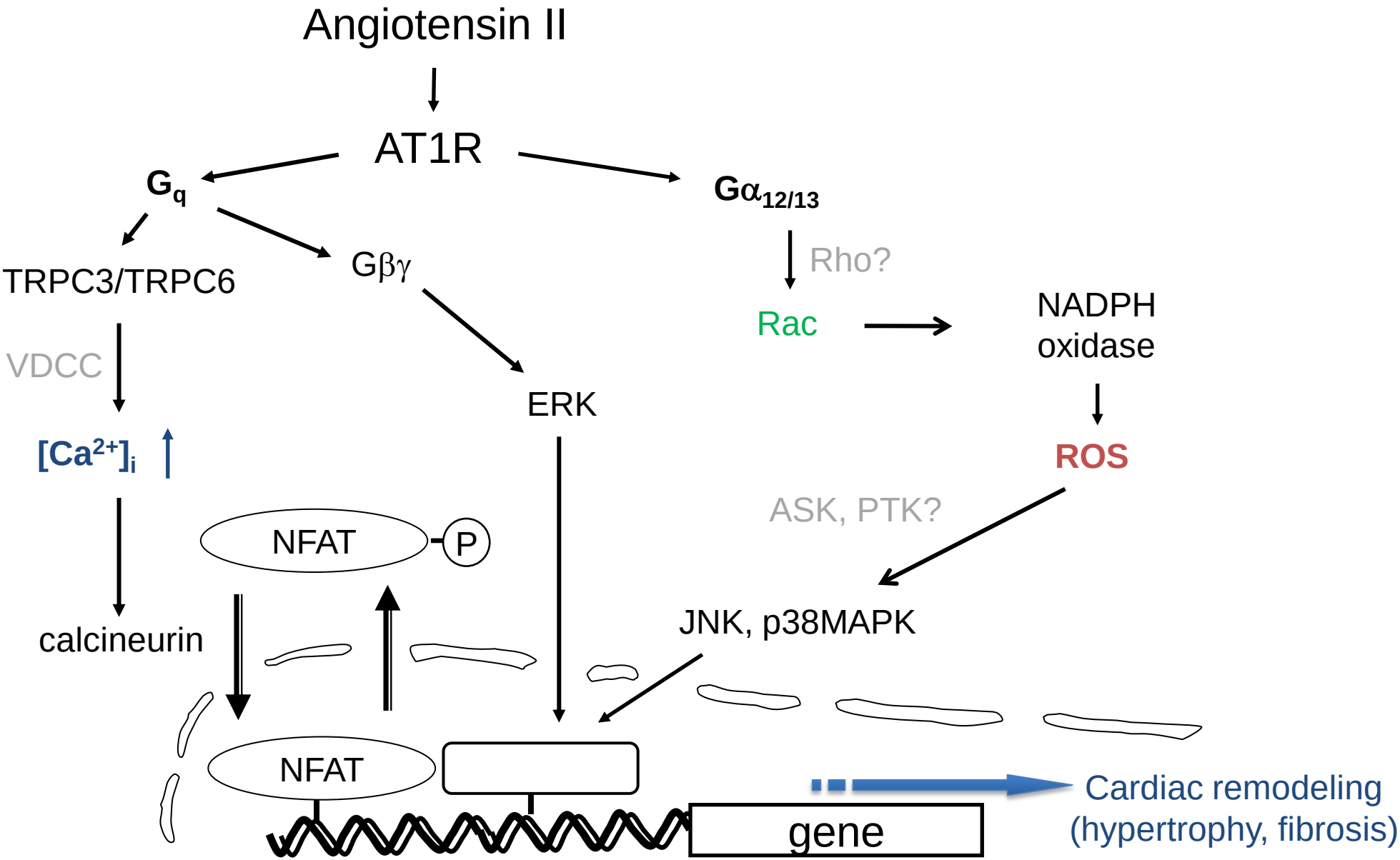

Figure 1. Nishida et al.

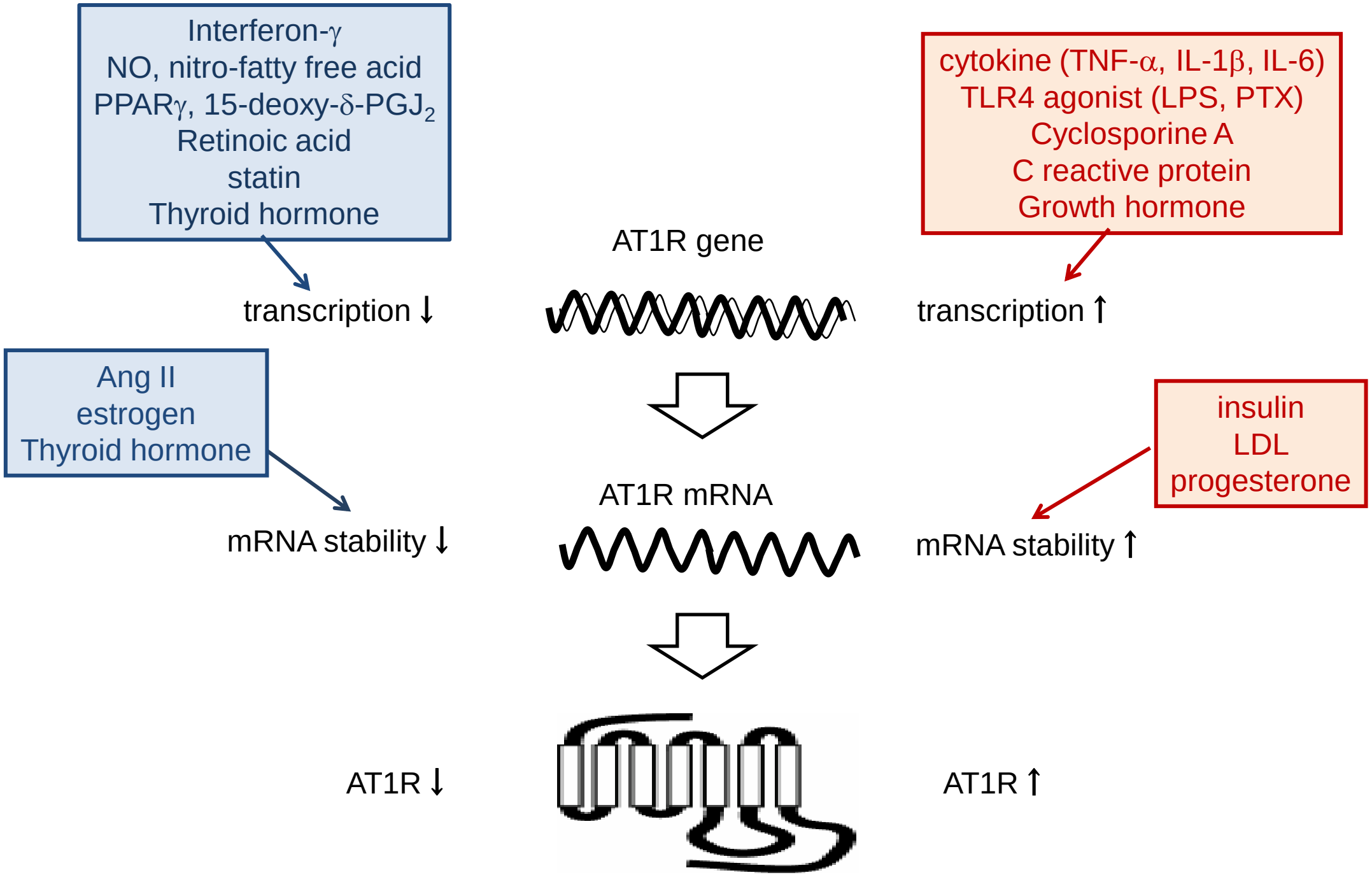

Figure 2. Nishida et al.

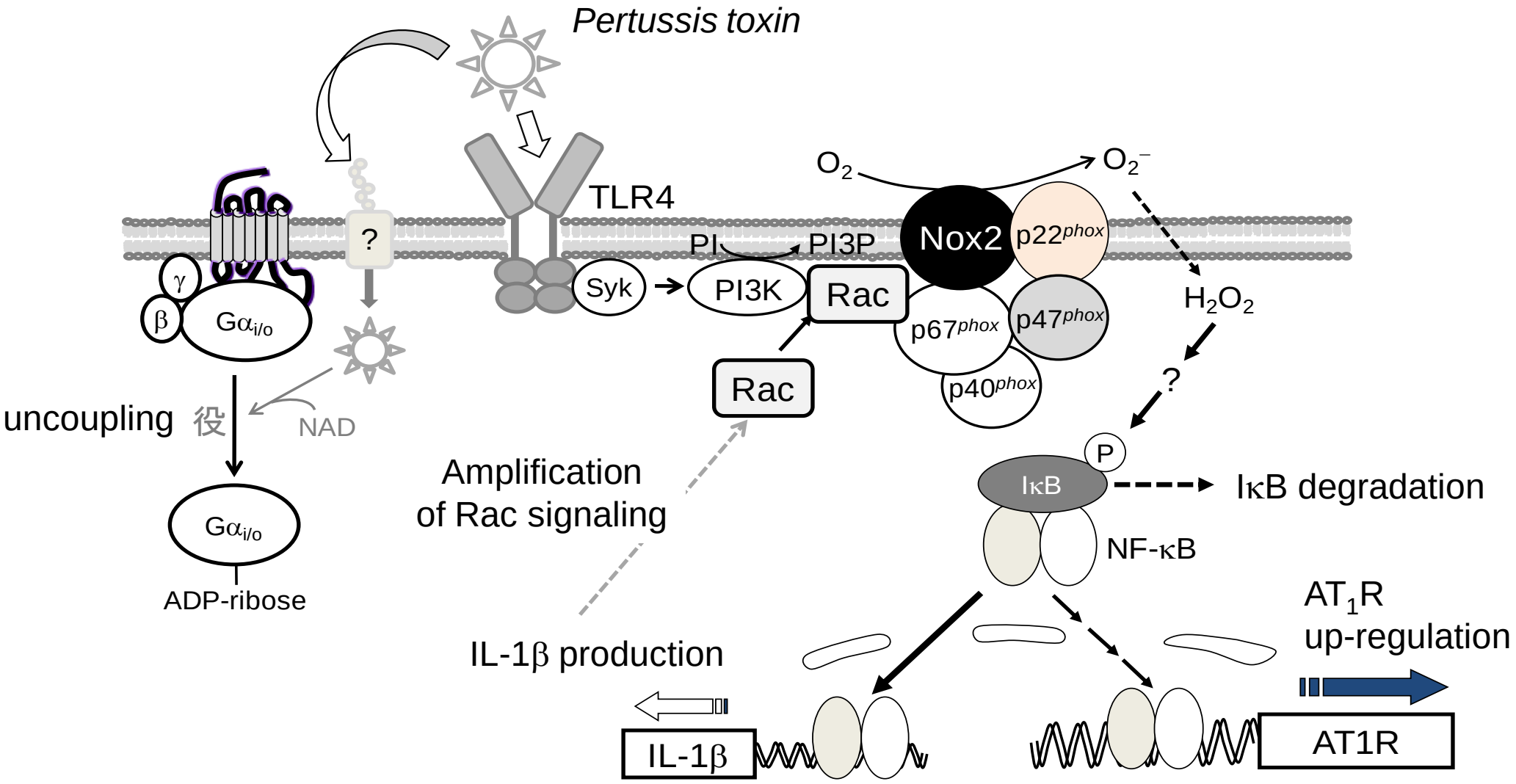

Figure 3. Nishida et al.

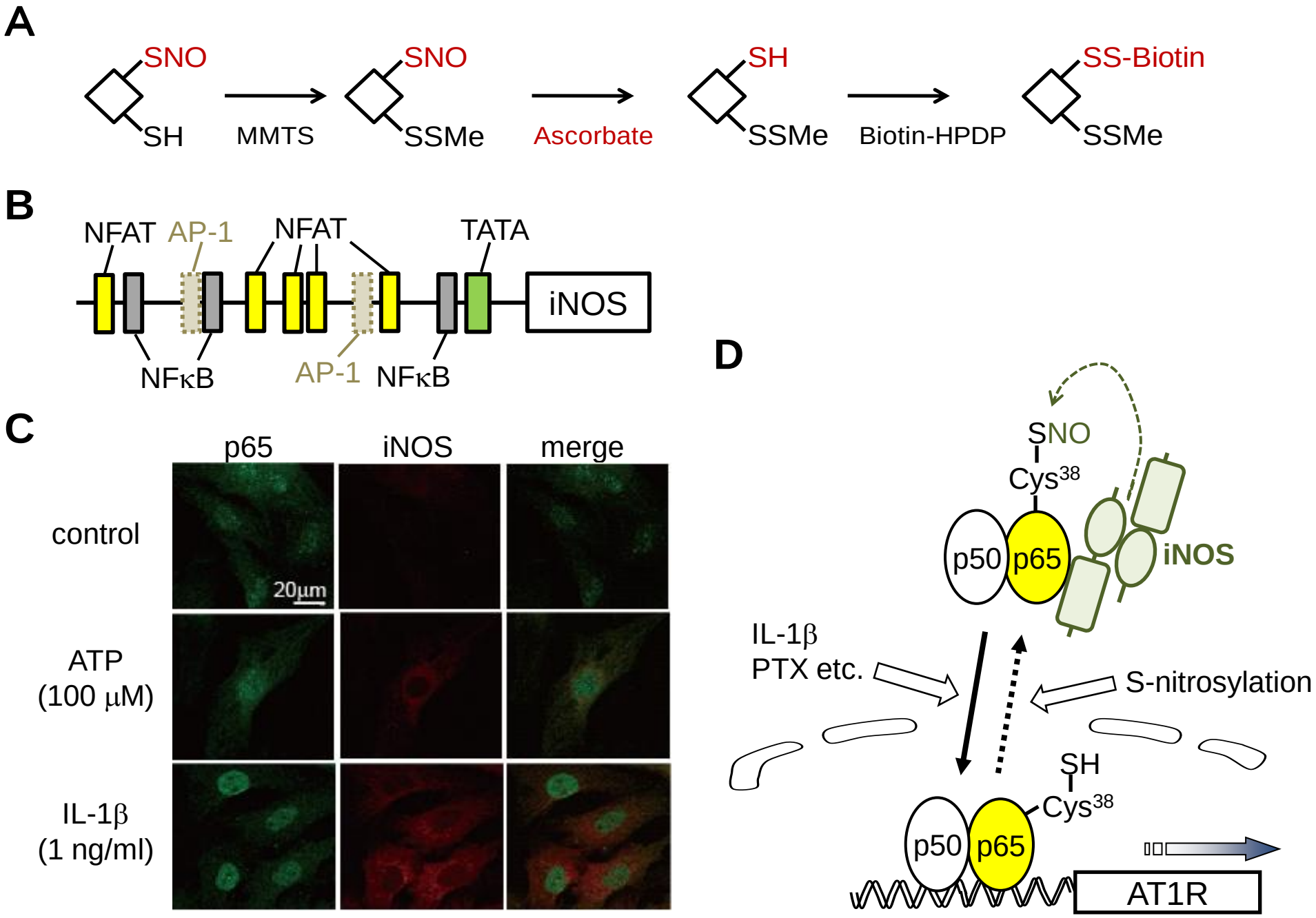

Figure 4. Nishida et al.