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Shear stress-induced Ang II AT1 receptor activation: G-protein dependent and independent mechanisms

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ABSTRACT

Mechanotransduction enables cells to sense and respond to stimuli, such as strain, pressure and shear stress (SS), critical for maintenance of cardiovascular homeostasis or pathological states. The angiotensin II type 1 receptor (AT1R) was the first G protein-coupled receptor described to display stretch-induced activation in cardiomyocytes independent of its ligand Ang II. Here, we assessed whether SS (15 dynes/cm^2, 10 min), an important mechanical force present in the cardiovascular system, activates AT1R independent of its ligand. SS induced extracellular signal-regulated kinase (ERK) activation, used as a surrogate of AT1R activation, in Chinese hamster ovary cells expressing the AT1R (CHO + AT1) but not in wild type cells (CHO). AT1R dependent SS-induced ERK activation involves Ca^{2+} inflow and activation of Gαq since Ca^{2+} chelator EGTA or Gαq-specific inhibitor YM-254890 decreased SS-induced ERK activation. On the other hand, the activation of JAK-2 and Src, two intracellular signaling molecules independent of G protein activation, were not differently modulated in the presence of AT1R. Also, ERK activation by SS was observed in CHO cells expressing the mutated AT1R DRY/AAY, which has impaired ability to activate Gαq dependent intracellular signaling. Altogether we provided evidence that SS activates AT1R in the absence of its ligand by both a G protein-dependent and -independent pathways. The biological relevance of these observations deserves to be further investigated since the novel mechanisms described extend the knowledge of the activation of GPCRs independent of its traditional ligand.

1. Introduction

AT1. angiotensin receptors (AT1R) are members of the G protein-coupled receptor (GPCR) family, which bind the angiotensin II (Ang II) and elicit intracellular effects. Although the signaling pathways of AT1R has been well studied during the last 20 years, there must be novel aspects to be uncovered as suggested by the recent discovery of biased ligands of AT1R [1,2]. Classically, agonist stimulation of AT1R causes the activation of heterotrimeric G proteins leading to activation of protein kinase C and increase in cytoplasmic calcium concentration. However, stimulation of the receptor also leads to additional signaling mechanisms non-G protein related, such as activation of the Janus kinase-signal transducers (JAK2) and c-Src [1,3].

Mechanochemical signal transduction originates at the cell membrane, and several candidate sensor molecules have been postulated, including ion channels, tyrosin kinase receptors, G-proteins, enzymes, integrins, and proteins from the cytoskeleton [4], however the molecular mechanisms underlying downstream events and their contribution for physiologic and pathologic states remain largely unknown.

Recent work suggests that load-induced membrane stretch on cardiomyocytes activates AT1R signaling independent of its ligand Ang II [5–7]. Mechanical transduction in the heart tissue by AT1R activates GRK5 and GRK6 which promotes AT1R internalization and β-arrestin recruitment. The formation of AT1R-β-arrestin complex by mechanical stretch induces ERK signaling in a manner that does not require G-proteins or other second messenger generation [8]. The activation of this particular subset of intracellular signaling of the AT1R by mechanical stretch has been proven to be beneficial for *in vivo* cardiovascular function [9–13].

In this context, endothelial cells lining the cardiovascular system are also exposed to shear stress (SS), the frictional force of

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blood flow on the vessel wall, which has been associated with anti-inflammatory and anti-atherogenic properties [4].

In the present work we tested the hypothesis that the AT1R can be activated by SS independent of its ligand. We used ERK phosphorylation as a downstream surrogate of AT1R activation since ERK is activated by dependent and independent G-proteins elicited by AT1R. Our data show that AT1R can be activated by SS an effect that requires both G protein-dependent and -independent intracellular pathways and results in ERK activation in Chinese hamster ovary (CHO) cells that stably express the AT1R.

2. Methods

2.1. Cell culture

CHO cells were grown in 100 mm culture dishes in Dulbecco's Modified Eagle Medium (DMEM), high-glucose culture medium supplemented with L-glutamine, antibiotic (penicillin and streptomycin), HEPES buffer, and 10% FBS. Cells were routinely subcultured using trypsin–EDTA to mobilize them. For transfection, CHO cells were plated in 60 mm dishes 1 day prior to transfection and the selection of clones that stably expressed the wild-type AT1 receptor (CHO + AT1) or the mutated AT1 receptor (CHO + AT1–DRY/AAY) was performed. Mutated AT1 receptor DRY/AAY is severely impaired in $G_{\alpha q/11}$-coupling and has no detectable IP3 accumulation [14]. The plasmid was kindly provided by Prof. Laszlo Hunyady from Semmelweis University, Hungary.

Angiotensin II was purchased from Sigma and the JAK-2 inhibitor, AG490, from Calbiochem. Candesartan was a kind gift from AstraZenica (São Paulo, SP, Brazil). The specific $G_{\alpha q/11}$-selective protein inhibitor YM-254890 was provided by Astellas Pharma. The dose used of each compound is described in the figure legends.

2.2. Shear stress protocol

CHO cells were subjected to controlled SS for 10 min as previously described by our group [15,16]. Cells were plated in 100 mm dishes precoated with 1% gelatin and serum-starved for 24 h before shearing. SS at 15 dynes/cm^2 was produced by a cone plate viscometer. At the end of the experiment, the cells were washed with cold phosphate-buffered solution and lysed for further analysis.

2.3. Western Blot analysis

Cells were lysed in lysis buffer (1 mM EDTA, 1 mM EGTA, 2 mM $MgCl_2$, 5 mM KCl, 25 mM HEPES, 1 mM PMSF, 2 mM DTT, 0.1%Triton X-100, and protease and phosphatase inhibitor cocktail; Sigma–Aldrich). After 10 min on ice, samples were centrifuged at 10,000*g* for 10 min to remove cellular debris. Cell lysate (5–40 μg) supernatants were heated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer, fractioned by SDS–PAGE, and transferred to Hybond membranes (GE Healthcare-United Kingdom). Transfer efficiency was monitored by 0.5% Ponceau S staining. The blotted membranes were first blocked with 5% non-fat milk and proteins were then detected using their respective antibodies. The phosphorylated ERK (pERK), pSrc, pJAK2, and ERK antibodies were obtained from CellSignaling (1:1000; Beverly, MA, USA). Signals were detected using an ECL detection kit (GE Healthcare).

2.4. Gene expression by RT-PCR

Total RNA was isolated with Trizol Reagent according to the manufacturer's instructions and cDNA synthesis was performed with radom hexamers (High Capacity cDNA Archive kit-PE Applied Biosystem). The reaction was carried out using Taq polymerase The PCR products were analyzed by electrophoresis on agarose gel. The

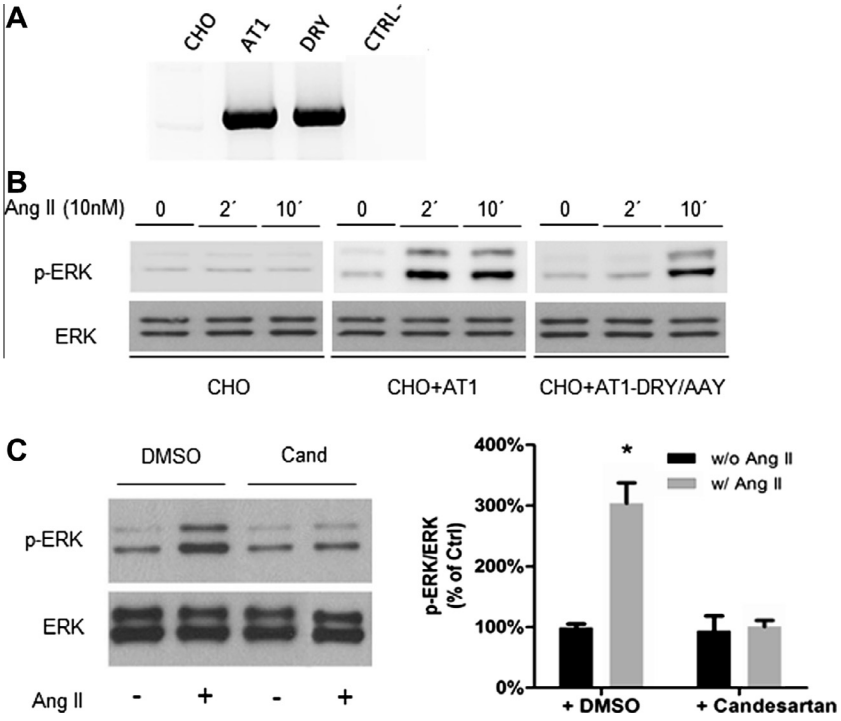

Fig. 1. CHO cells used to dissect the SS-induced response in CHO cells. (A) Analysis of AT1R constructs expression in CHO cells by PCR. (B) Representative western blotting of time-response p-ERK activation upon angiotensin II 10^{-6} M stimulation. (C) Blockade by Candesartan of Ang II-induced p-ERK activation in CHO + AT1R cells. Each bar represents the mean ± SEM of five experiments. $^*p < 0.05$, compared with control.

primers used to AT1 receptor were: antisense 5′-GGAAAC AGCTTGGTGGTGAT-3′, sense 5′-ACATAGGTGATTGCCGAAGG-3′.

2.5. Statistical analysis

All data represent at least four independent experiments. Numerical data are expressed as mean ± SEM. Comparisons among groups were performed using Student's *t*-test and two-way analysis of variance (ANOVA) followed by the Tukey *post hoc* test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. AT1 receptors are activated by shear stress

The CHO cell model system was used to dissect the SS-induced responses since it enables precise and consistent genetic manipulations of the renin–angiotensin system with activation of downstream signaling without background effects associated with endogenous components of the RAS [17]. CHO were genetically modified to permanently express the wild type or mutated AT1R molecules lacking the coupling to $G_{\alpha q/11}$ protein. Each cell lineage was confirmed by both mRNA expression and ERK activation upon angiotensin II stimulation. Fig. 1A shows that CHO cells lack AT1R mRNA expression while its expression was only observed on both transfected CHO cells lineage (CHO + AT1 and CHO + AT1–DRY/AAY). Functionally, CHO cells did not activate p-ERK upon stimulation to angiotensin II (10^{-6} M, 10 min) while CHO + AT1–DRY/AAY cells lack the fast (2 min) activation of p-ERK via G protein-dependent pathway as previously described [18], which confirm its impaired $G_{\alpha q/11}$-coupling ability (Fig. 1B). Fig. 1C shows the efficacy of the Angiotensin Receptor Blocker (ARB) candesartan to block AT1R activation by angiotensin II in CHO + AT1 cells.

CHO-AT1displayed SS-induced (15 dynes/cm^2) ERK activation (291 ± 26%) (Fig. 2A) which was completely inhibited by candesartan (107 ± 9%), an inverse agonist ARB (Fig. 2B), whereas wild type CHO cells failed to respond (105 ± 7%) (Fig. 2A) indicating that the presence of the AT1R is necessary and sufficient for SS-induced ERK activation in CHO cells.

To further investigate the ability of candesartan to block AT1R in CHO + AT1 cells, we stimulated the cells with Ang II and SS independently or concurrently (Fig. 2C). Ang II and SS independently induced similar ERK activation in CHO + AT1 cells (Ang II, 250 ± 26%; SS, 242 ± 16%). When both stimuli were combined,

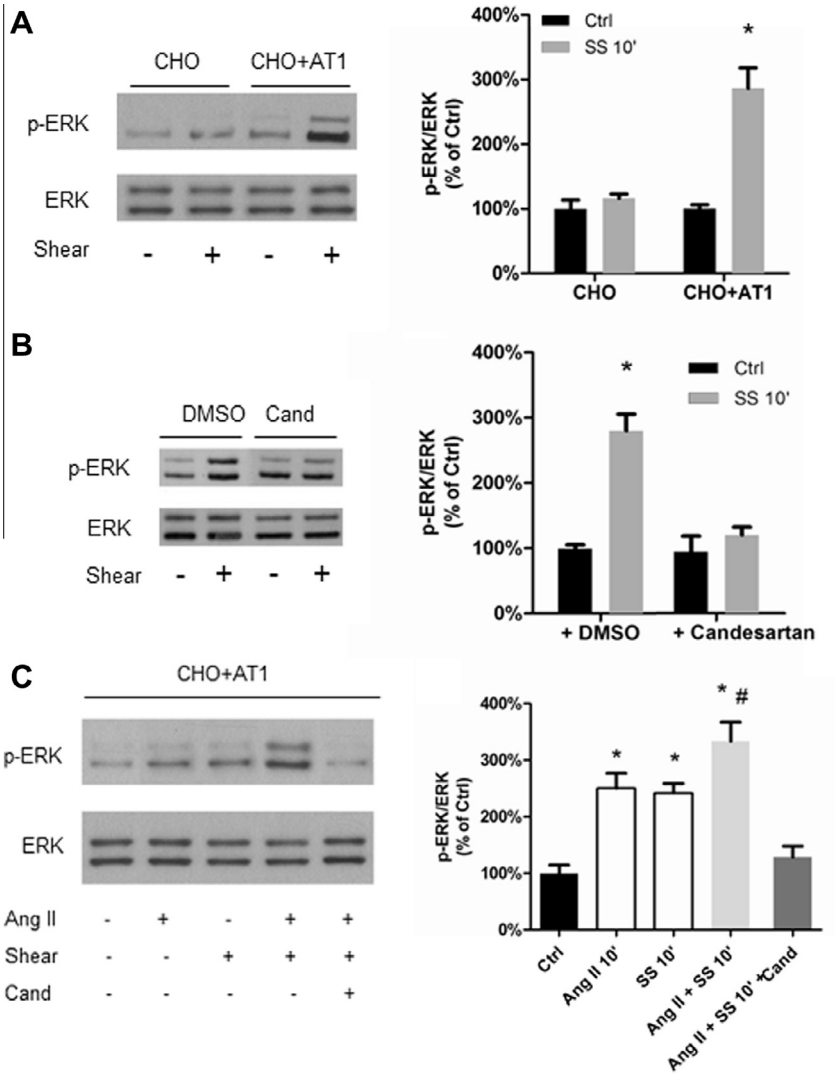

Fig. 2. (A) Effects of shear stress (15 dynes/cm^2) for 10′ on p-ERK activation in CHO and CHO + AT1R cells, and (B) in the presence of candesartan in CHO + AT1R cells. (C) Effects of Ang II and SS on p-ERK activation when applied concomitantly. Each bar represents the mean ± SEM of four to six experiments. $^{*}p < 0.05$, compared with control; $^{\#}p < 0.05$, compared with shear stress or Ang II.

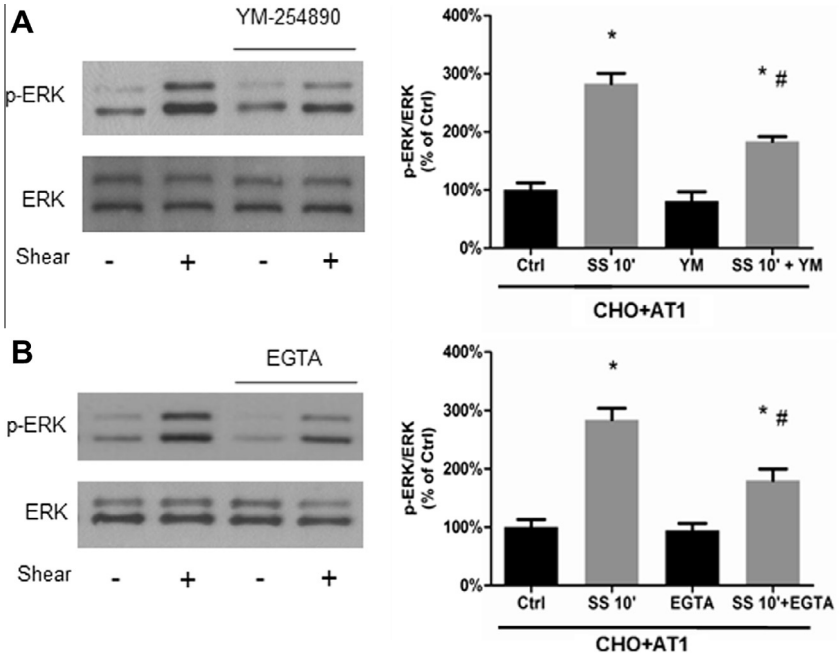

Fig. 3. Effects of (A) the protein Gαq inhibitor YM-254890 (100 nM) and (B) EGTA on CHO + AT1 cells subjected to shear stress for 10 min. Each bar represents the mean ± SEM of four to six experiments.*$p < 0.05$, compared with control;#$p < 0.05$, compared with shear stress + EGTA.

greater ERK activation occurred than either stimulus alone (333 ± 32%). Under the combined condition, candesartan completely abrogated ERK signaling (128 ± 19%). Altogether, the data showed that the AT1R is directly activated by SS even in the presence of its ligand Ang II and candesartan inhibited ERK activation mediated by both Ang II and SS.

3.2. Evaluation of G protein-dependent pathway in shear stress-induced ERK activation via AT1R

To investigate the role of *Gαq-protein* in SS-induced ERK activation, we pre-treated CHO + AT1 cells with the G*αq*-protein inhibitor YM-254890 (100 nM) before SS (10 min) (Fig. 3A). YM-254890 treatment caused a significant reduction in SS-induced ERK activation (SS, 282 ± 17%; SS + YM-254890, 173 ± 9%) without interfering with ERK signaling in control static cells. Consistent with these observations, the administration of the calcium chelator EGTA (Fig 3B) significantly reduced SS-induced ERK activation (SS, 284 ± 19%; SS + EGTA 183 ± 8%). These data suggest that SS-induced ERK activation via AT1R requires, at least in part, the activation of the Gαq and the downstream second messenger Ca^{2+}.

3.3. Evaluation of G protein-independent pathway in shear stress-induced ERK activation via AT1R

c-Src and JAK-2, two likely candidates involved in AT1R-mediated ERK activation by Ang II, were activated in both CHO cells (130 ± 10.5% and 128 ± 8.3%, respectively) and CHO + AT1 cells in response to SS (127 ± 5.9% and 141 ± 8.1%, respectively) (Fig. 4A and B), whereas ERK activation was observed only in CHO + AT1 cells (Fig. 2A) suggesting that SS-mediated ERK activation via AT1R is not dependent on activation of c-Src or JAK-2.

Furthermore, JAK-2 has been shown to activate ERK after 15 min of mechanical stretch in CHO + AT1 cells [7]. We used a second approach, namely a specific JAK-2 inhibitor, AG490 (50 μM, Calbiochem), which efficiently inhibited JAK-2 activation induced by SS. As expected, SS-induced ERK activation was not abrogated by the AG490 inhibitor (SS, 286 ± 21.6%; SS + AG490, 279 ± 34.2%), confirming that JAK-2 modulation by SS does not require the AT1R and that neither c-Src nor JAK-2 appear to be involved in AT1R-dependent ERK activation induced by 10 min SS.

To further evaluate the G protein-independent pathway, CHO + AT1–DRY/AAY were also submitted to SS. Fig. 4C shows that even in the absence of any G protein-coupling, ERK was still partially activated upon stimulation to SS (CHO + AT1, 252 ± 20.7%; CHO + AT1–DRY/AAY, 149 ± 12.2%) suggesting that another intracellular pathway other than JAK-2 and c-Src can activate ERK signaling, independently of the G-protein pathway.

4. Discussion

The AT1R is a member of the G-protein-coupled receptor (GPCR) family, which mediates most of the actions of Ang II in the cardiovascular system. Upon agonist binding, AT1R activate a broad range of downstream signaling pathways. In the present study, we provide novel evidence that SS activates AT1R, independent of its ligand Ang II. The SS-induced response requires both G protein-dependent and G protein-independent pathways other than JAK-2 and cSrc. Thus, the SS-induced response appears to differ from the one elicited by mechanical stretch since the downstream events here described are not the same. Future studies assessing the precise conformational change of the AT1R associated with SS and stretch may shed additional light on this issue.

The findings in the present study were obtained in CHO cells permanently transfected with the wild type AT1R or mutated AT1R–DRY/AAY. CHO cells in culture are robust; do not express the AT1R and upon transfection with the receptor recapitulated specific features of the endogenous system enabling careful dissection of the response with consistent pattern.

Our findings support the hypothesis that transmembrane receptors can act as mechanosensors independently of their ligand. SS has been shown previously to ligand-independently activate tyrosine kinase receptors, such as vascular endothelial growth factor receptor 2, and induce eNOS activation in response to flow [19]. More recently, the formyl peptide receptor, a GPCR, was shown to be a mechanosensor of fluid SS, which induces constitutive

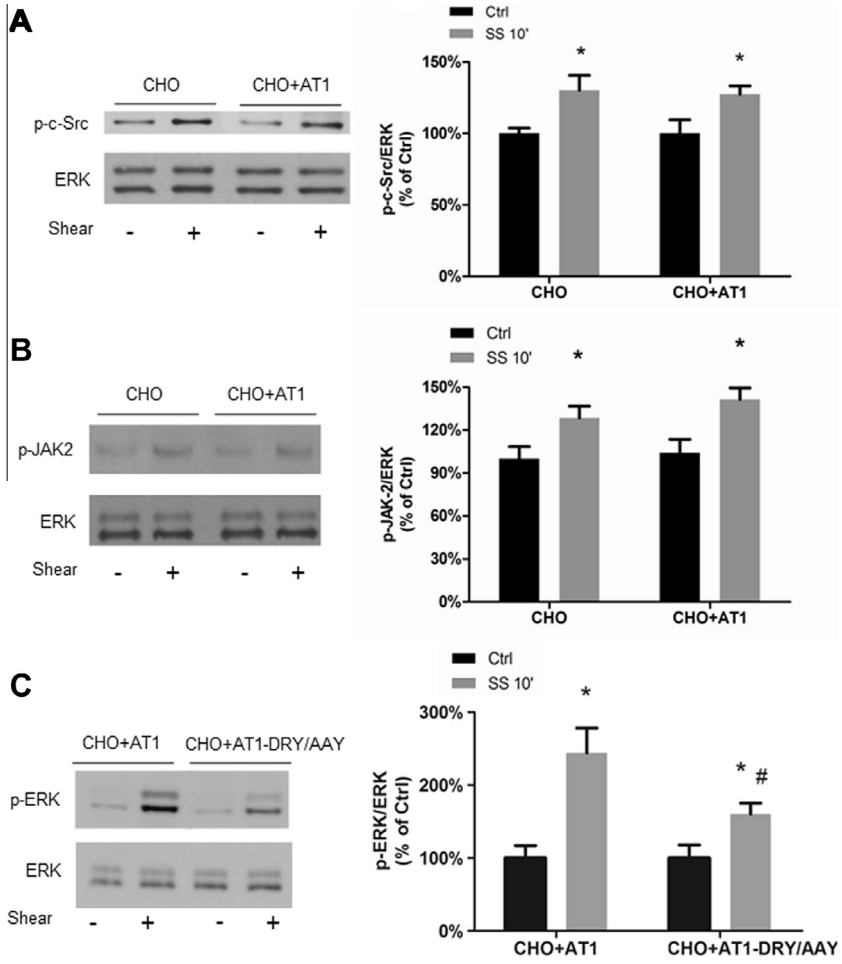

Fig. 4. Effects of shear stress (15 dynes/cm^2) for 10′ on (A) c-Src and (B) JAK-2 activation in CHO and CHO + AT1 cells. (C) Effects of shear stress on p-ERK activation in CHO + AT1–DRY/AAY cells. Each bar represents the mean ± SEM of four to six experiments. $^*p < 0.05$, compared with control cells.

activity inhibition and reduces pseudopod projection in HL-60 cells, a mechanism by which SS regulates leukocyte activation and cell adherence and migration [20].

Upon ligand binding to GPCRs, the G-protein complex dissociates and releases α subunits from βγ subunits, promoting intracellular signaling [22]. The AT1R signal transduction pathway is mediated mainly by $G_{q/11}$-proteins which increase intracellular Ca^{2+} and activate protein kinase C. SS-dependent AT1R activation via classical G-protein signaling occurred since there was blockade of the response by either EGTA or Gαq-specific inhibitor YM-254890. Interestingly, AT1R also activate proteins other than the traditional heterotrimeric G proteins—a ''non-classical'' pathway usually associated with growth factors and cytokine receptors (e.g., tyrosine phosphorylated proteins, JAK kinase family, Src kinase family, and transactivation of platelet-derived growth factor receptor, epidermal growth factor receptor, and insulin growth factor receptor) [23,24].

Receptor activation also causes the desensitization and subsequent sequestration of AT1R with a b-arrestin-dependent process. However, during the process of AT1R desensitization, b-arrestin may also act as a scaffold for activation of other molecules such as AKT, PP2A and JNK [25]. Each of these three pathways converges to ERK1/2 activation, which was thus used as a surrogate of AT1R SS-mediated activation in the absence of Ang II. Our results showed that SS-induced c-Src and JAK activation occur independent of the AT1R under the present experimental conditions. In contrast, Zou et al. [7] demonstrated that AT1R activation induced by mechanical stretch, other important type of mechanical stimulus influencing the cardiovascular system, elicited the JAK-STAT pathway followed by ERK1/2 activation. Is important to note that β-arrestin 2 has been described as the main effector of AT1R response to mechanical stretch [6,8] and it may explain the residual p-ERK activation in response do shear stress in CHO + AT1-DRY/AAY cells and thus deserves to be furtherexplored in future studies.

The described G protein-dependent and -independent mechanisms highlight the complex interplay between the AT1R SS-induced responses that may influence endothelial cells, even in the absence of the ligand. Thus, it is tempting to speculate that the SS-induced response may potentially influence the therapeutic profile of different ARBs widely used for cardiovascular therapy. In this context, it will be important to establish the potential role of shear-stress acting directly on the Ang II AT1R to counteract the capacity of Ang II to impair endothelium-dependent NO-mediated dilatation via increase superoxide production [26]. This type of finding, together with other evidences [5,7,8,15,21] adds an additonal level of complexity in the understanding of the complex interplay between the components of the RAS and physical forces.

Altogether, we provide evidence for mechanosensitive ligand independent properties of the AT1R in response to SS that activate unique downstream pathways, which may elicit different cell phenotypes in the cardiovascular system.

Acknowledgments

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