FAILURE OF ANGIOTENSIN II AND INSULIN TO STIMULATE TRANSFORMING GROWTH FACTOR-\(\beta1\) RELEASE FROM CULTURED BOVINE RETINAL PERICYTES


SUMMARY - Background: Activation of the renin-angiotensin system (RAS) may induce cardiovascular and renal fibrosis in hypertension and diabetes. This fibrogenic effect is mainly mediated by Transforming Growth Factor-\(\beta1\) (TGF-\(\beta1\)), a multifunctional cytokine released by endothelial, vascular smooth muscle and renal mesangial cells, that is able to increase extracellular matrix deposition. Retinal capillary pericytes have functions similar to those of mesangial cells, including ability to synthesize and release TGF-\(\beta1\) and produce extracellular matrix. An intraocular RAS was described in the human eye and may produce effects similar to those observed in the heart and kidney, which could be mediated by TGF-\(\beta1\). In particular, TGF-\(\beta1\) might be involved in thickening of the capillary basement membrane in diabetic microangiopathy. We therefore aimed at evaluating the possible effects of Angiotensin-II on TGF-\(\beta1\) secretion by cultured retinal pericytes (BRP).

Methods: BRP cultures were incubated with Angiotensin-II or insulin (known to play a permissive effect on TGF-\(\beta1\) secretion by mesangial cells) or Angiotensin-II+insulin at final concentrations of \(10^{-10}\), \(10^{-8}\), \(10^{-6}\), \(10^{-5}\) mol/L.

Results: Baseline TGF-\(\beta1\) concentrations in the supernatants of pericyte cultures were \(6.139 \pm 1.919\) pg/mL/\(10^6\) cells; no changes of TGF-\(\beta1\) concentrations resulted from adding increasing amounts of Ang II, insulin or both.

Conclusions: Though confirming that cultured bovine retinal pericytes spontaneously release TGF-\(\beta1\), Angiotensin-II did not produce any stimulatory effects of in our experimental system.

Key-words: angiotensin II, transforming growth factor-\(\beta1\), retinal pericytes, insulin, diabetes.

RÉSUMÉ - Incapacité de l’Angiotensine II et de l’insuline à stimuler la libération de Transforming Growth Factor-\(\beta1\) par des péricytes bovins in culture.

Contexte : L’activation du système rénine angiotensine (SRA) peut induire une fibrose cardiovasculaire et rénale au cours de l’hypertension et du diabète. Cet effet fibrosant est principalement médié par le Transforming Growth Factor-\(\beta1\) (TGF-\(\beta1\)), une cytokine multifonctionnelle libérée par l’endothélium, les fibres musculaires lisses et les cellules mésangiales rénales, et capable d’augmenter les dépôts de matrice extracellulaire. Les péricytes des capillaires rétinien ont des fonctions similaires à celles des cellules mésangiales, dont la capacité à synthétiser et libérer du TGF-\(\beta1\) et produire de la matrice extracellulaire. Un SRA intraoculaire a été décrit dans l’œil humain et peut produire des effets similaires à ceux observés dans le cœur et le rein, qui pourraient être médiiés par le TGF-\(\beta1\). En particulier, le TGF-\(\beta1\) pourrait être impliqué dans l’épaississement de la membrane basale capillaire au cours de la microangiopathie diabétique. Ainsi, notre but était d’évaluer les effets possibles de l’angiotensine II sur la sécrétion de TGF-\(\beta1\) par des péricytes rétinien en culture (BRP).

Méthodes : Les cultures de BRP ont été incubées en présence d’angiotensine II ou d’insuline (connue pour jouer un effet permissif sur la libération de TGF-\(\beta1\) par les cellules mésangiales) ou d’angiotensine II+insuline à des concentrations finales de \(10^{-10}\), \(10^{-8}\), \(10^{-6}\), \(10^{-5}\) mol/L.

Résultats : Les concentrations basales de TGF-\(\beta1\) dans le surnageant de culture de péricytes étaient de \(6.139 \pm 1.919\) pg/Ml/\(10^6\) cellules : aucun changement de concentration de TGF-\(\beta1\) n’a été observé après addition de quantités croissantes d’Ang II, d’insuline ou des deux.

Conclusions : Bien que nous confirmions que les péricytes rétinien en culture libèrent spontanément du TGF-\(\beta1\), nous n’observons pas d’effets stimulants de l’Angiotensine II dans notre système expérimental.

Mots-clés : angiotensine II, transforming Growth Factor-\(\beta1\), péricytes rétinien.

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The renin-angiotensin system (RAS) plays a pivotal role in the regulation of cardiovascular homeostasis through the well known vasoconstricting and aldosterone-stimulating effects of its main effector, circulating angiotensin II (Ang II) [1]. More recently, other important cardiovascular and renal effects of the RAS have been described, including growth regulation of smooth muscle cells [2] and synthesis of extracellular matrix components [3]. Local tissue RASs, described over the last few years [4], may be responsible for these effects and might be involved in the pathogenesis of cardiovascular and renal damage secondary to hypertension and diabetes [1, 2]. In particular, chronic uncontrolled RAS hyperactivity in these conditions might result in myocardial fibrosis and atherosclerosis [1-4].

Ang-II promotes the secretion of a number of growth factors [2-6] which may mediate its effects. Transforming Growth Factor-β (TGF-β), a family of multifunctional cytokines regulating extracellular matrix deposition [7], smooth muscle cell proliferation and endothelial cell function [8] are among its most important mediators. Ang-II may induce myocardial fibrosis and thickening of the vessel wall in hypertension by increasing the production of TGF-β1 [2, 9]. Ang-II may also stimulate mesangial cells to release TGF-β1 which, in turn, promotes the synthesis of matrix proteins, such as laminin, fibronectin and type IV collagen [10], thus favouring glomerular fibrosis and permeability [15], endothelial cell growth and differentiation [14] and the synthesis of extracellular matrix [16]. Such functions are similar to those of pericytes, uniquely present in a 1:1 ratio to endothelial cell [14]. Pericytes appear to have multiple functions, including the regulation of vascular tone, flow and permeability [15], endothelial cell growth and differentiation [14] and the synthesis of extracellular matrix [16]. Such functions are similar to those of mesangial cells in the kidney, and include the ability to synthesize and release TGF-β1 [17]. Circulating levels of TGF-β1 are higher in patients with than in those without retinopathy [18]. Hence, TGF-β1 might be involved in thickening of the capillary basement membrane in diabetic microangiopathy.

An intraocular RAS has been described in the human eye [19, 20], suggesting that it might produce effects similar to those observed in the heart and kidney, which could be mediated by TGF-β1. Circulating and intraocular levels of Ang-II, prorenin and angiotensin-converting enzyme (ACE) were found to correlate with the severity of diabetic retinopathy [21] and treatment with an ACE-inhibitor was reported to reduce progression of this complication [22].

This study was aimed at further clarifying the role of the RAS and TGF-β in the pathogenesis of diabetic retinopathy, by evaluating the possible effects of Ang-II on TGF-β1 secretion by cultured retinal pericytes (BRP). Insulin, alone and in combination with Ang-II, was also evaluated, since it was described to have a permissive effect on TGF-β1 release from mesangial cells [10].

**MATERIALS AND METHODS**

BRP were obtained from bovine retinas, with a partial modification of the method of Wong et al. (1987) and McIntosh et al. (1988) as previously described [23]. They were grown in DMEM 5.6 mmol/L glucose (Sigma, St. Louis) with 20 % FCS in primary culture. BRP in primary culture were detached by trypsin-EDTA and seeded in 6-well plates (approximately 1×10⁵ cells/well, 12,500 cells/cm²) for secondary cultures, using DMEM with 5.6 mmol/L glucose and 10 % FCS. Once confluent again, the media were removed, the cells rinsed twice with Phosphate Buffered Saline (PBS - Sigma) and similar media without FCS containing Ang II (Sigma,) or insulin or Ang II + insulin at final concentrations of 10⁻¹⁰, 10⁻⁸, 10⁻⁶ mol/L were added. Control wells without Ang II or insulin were run in the relevant plates.

Twelve hours later, the supernatants were collected for TGF-β1 assay. The latter was performed by a commercial ELISA (TGF-β1 Immunoassay, Quantikine, R & D Systems) with sensitivity less than 7 pg/mL. Intra- and interassay variations of the assay were 6.1 % and 11.4 %, respectively. The concentrations measured in the supernatants were corrected by the numbers of cells in the wells, as counted manually in Bürker chambers.

**Statistics**

Results are expressed as mean ± standard deviation (pg/mL/10⁶ cells). Statistical analysis was done by ANOVA followed by Bonferroni’s test. In consideration of the multiple comparisons performed, the significance level was set at p < 0.01.

**RESULTS**

TGF-β1 concentrations were 6139 ± 1919 pg/mL/10⁶ cells in the supernatants of pericyte cultures to which no Ang II had been added. TGF-β1 levels were not significantly modified by any of the Ang II concentrations added (Fig. 1).
In the second series of experiments, TGF-β1 concentrations were 4404 ± 1065 pg/mL/10^6 cells in the supernatants of wells to which no insulin had been added. Also in this case, no changes of TGF-β1 concentrations resulted from the addition of increasing amounts of insulin (Fig. 1).

The simultaneous addition of both Ang II and insulin (10^{-10}, 10^{-8}, 10^{-6}, 10^{-4} mol/L) did not induce
concentrations of TGF-β1 different from those observed in the presence of either hormone alone or in the absence of both (baseline values 5372 ± 1214 pg/mL/10⁶).

## DISCUSSION

Although the RAS is part of a complex network aimed at regulating cardiovascular homeostasis in health, its chronic uncontrolled activation in hypertension and diabetes may cause cardiovascular and renal damage through mechanisms that may involve TGF-β1 [1, 2, 7]. This paper aimed at investigating whether, similarly to what observed in mesangial cells [10], Ang II could stimulate the release of TGF-β1 from pericytes, thus possibly contributing to damage retinal capillaries in diabetes. Though confirming that cultured bovine retinal pericytes are able to spontaneously release TGF-β1, we were unable to observe any stimulatory effects of Ang II in our experimental system. This could be due to a number of reasons, including binding specificity of Ang II, strength of the stimulus applied, or involvement of other members of the TGF-β family. Pericytes are likely to have specific receptors for angiotensin because their release of VEGF after stimulation with Ang II is inhibited by specific AT-1 receptor blockers [24]. The fact that the final concentrations used in vitro ranged from lower to far higher than physiological circulating levels of Ang II rules out the possibility that pericytes were adequately stimulated in our system. The possibility that release of TGF-β2 rather than TGF-β1 is selectively stimulated by Ang II in BRP cannot be ruled out. Katsura et al. [25] showed that pericytes are able to release this member of the TGF-β family, which could have autocrine/paracrine actions. However, most available data on the role of TGF-β in the development of vascular damage collected so far are concerned with TGF-β1 [2].

Ang II on its own may contribute to, but not be the principal regulator of, TGF-β1 release from pericytes. Since insulin was found to potentiate the effect of Ang II on TGF-β1 release from mesangial cells [10], we verified if it could exert similar effects on pericytes. Once again, we could not observe any stimulation of the release of TGF-β1 from BRP either in the presence of insulin alone or in combination with Ang II.

Pericytes may play a role in the pathogenesis of capillary damage through altered synthesis of basement membrane components [26] but these results suggest that increased release of TGF-β1 resulting from activation of the RAS and/or insulin may not be among the mechanisms involved. Treatment with ACE-inhibitors may reduce the progression of moderate to severe retinopathy [22] and blockade of the RAS was reported to prevent the growth of retinal new vessels in an animal model of retinopathy of prematurity [27]. Since pericytes disappear early and selectively from the capillary wall in the course of diabetic retinopathy [28] and most of them are likely to have disappeared from retinal capillaries once retinopathy has reached the stages which appear to be influenced by ACE-inhibition, the possibility remains that activation of an intraocular RAS is involved in initiating and/or maintaining pathogenic mechanisms in later stages of retinopathy [29]. This work was supported by a grant from Ministry of the University and Scientific and Technological Research.

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