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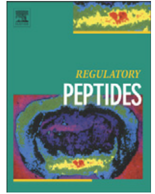

Rapid communication

Angiotensin II induces superoxide generation via NAD(P)H oxidase activation in isolated rat pancreatic islets

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ABSTRACT

Angiotensin II (Ang II) controls blood pressure, electrolyte balance, cell growth and vascular remodeling. Ang II activates NAD(P)H oxidase in several tissues with important function in the control of insulin secretion. Considering the concomitant occurrence of hypertension, insulin resistance and pancreatic B cell secretion impairment in the development of type II diabetes the aim of the present study was to evaluate the effect of ANG II on NAD(P)H oxidase activation in isolated pancreatic islets. We found that ANGII-induced superoxide generation via NAD(P)H oxidase activation and increased protein and mRNA levels of NAD(P)H oxidase subunits ($p47^{PHOX}$ and $gp91^{PHOX}$).

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1. Introduction

Angiotensin II (ANGII) is an active component of renin–angiotensin system (RAS). This octapeptide originally described as a potent vasoconstrictor, is now recognised as a multifunctional hormone influencing many important cellular processes for the regulation of vascular function, including cell growth, apoptosis, migration, inflammation and fibrosis [1,2]. ANGII is an important growth modulator of blood vessels and plays a critical role in regulating blood pressure and fluid homeostasis in physiological conditions. Through its vasoconstrictor, mitogenic, pro-inflammatory and pro-fibrotic actions, ANGII contributes to altered vascular tone, endothelial dysfunction, structural remodeling, and vascular inflammation, characteristic features of vascular damage in hypertension, atherosclerosis, vasculitis and diabetes [2–5]. The systemic RAS is composed of several components including the precursor angiotensinogen, ANGII, and multiple receptor subtypes [2], linked by alternative, non-exclusive components such as the enzymes renin and ACE (angiotensin-converting enzyme). ANGII induces its effects via at least two G-protein-coupled transmembrane receptors, ANGII receptor 1 (AT1) and ANGII receptor 2 (AT2), of which the former is considered to be more physiologically important [6].

The subcellular mechanisms and signaling pathways whereby ANGII mediates its physiological and pathophysiological effects are complex [2]. All vascular cell types, including endothelial cells, smooth muscle cells, adventitial fibroblasts, and resident macrophages, produce reactive oxygen species (ROS) [6]. Of particular importance in the vasculature are superoxide (O_2^-) and hydrogen peroxide (H_2O_2), since these ROS act as signaling molecules. The major source of ROS in the vascular wall is non-phagocytic NAD(P)H oxidase which is regulated by vasoactive agents, growth factors and mechanical forces [5]. The best characterized system in vascular cells is ANGII-stimulated NAD(P)H oxidase-mediated generation of O_2^-, which appears to be upregulated in hypertension, atherosclerosis and diabetes [5]. The components of NAD(P)H oxidase complex include the membrane bound cytochrome *b*558, composed of 2 subunits, $p22^{PHOX}$ and $gp91^{PHOX}$, and 4 cytosolic subunits, $p47^{PHOX}$, $p67^{PHOX}$, $p40^{PHOX}$ and the small GTP-binding protein, Rac1/Rac2 [7].

Metabolic syndrome identifies a condition in which several metabolic risk factors (e.g. insulin resistance, dyslipidemia, obesity, hypertension, hypercoagulation and hyperuricemia) are clustered, resulting in an increased risk of atherosclerotic cardiovascular disease. In addition, insulin resistance is a risk factor for impaired glucose tolerance and type 2 diabetes and has a tight relationship with obesity and hypertension [8]. The natural history of type 2 diabetes is characterized by progression of the disease severity. In early stages, patients generally exhibit peripheral insulin resistance and their pancreatic beta cells still produce insulin, but insufficient compensation for insulin resistance leads to glucose intolerance. As the disease progresses, most patients eventually exhibit pancreatic beta cell dysfunction or beta cell loss, often leading to severe impairment of insulin secretion.

Infusion of ANGII has been shown to induce insulin resistance, and ACE inhibitors and ANGII receptor blockers improve insulin sensitivity [8,9]. In insulin resistance states, blood vessels generate oxygen free

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Table 1
Primers used in the Real Time PCR

Primer	Sequence
p47 PHOX	Sense: CACCTTCATTCGCCACATCGC
	Antisense: ACGCTGCCCATCATCATACCACCTG
gp91 PHOX	Sense: GAAGGGATTCAGGATGGAGGTG
	Antisense: CTTCACTGGCTGTACCAAAGGG
G3PDH	Sense: AACCCATCACCATCTTCCAGG
	Antisense: ATACTCAGCACCAGCATCACCC

radicals in excess, which rapidly destroy NO (nitric oxide) and promote the production of hydrogen peroxide and free hydroxyl radicals that in turn cause lipid peroxidation and damage to cell membranes [8,9]. Short-term infusion of ANGII impairs first-phase insulin release, possibly through changes in intraislet blood flow [10]. However, the effects of chronic exposure of the pancreas to ANGII are unknown. Accumulating evidence has suggested that oxidative stress increases in pancreatic beta cells of animal models and patients with type 2 diabetes [11–14]. Since pancreatic islets have low intrinsic antioxidant capacity compared with other tissues [15], oxidative stress may be the main cause of beta cell damage. Recent report demonstrated that pancreatic beta cells expressed phagocyte-like NAD(P)H oxidase [16]. Just recently, we reported that exposure of beta cells to glucose, palmitic acid and IL1β result in increased expression of NAD(P)H oxidase components and subsequent production of ROS [17].

Considering all these data and that an intrinsic RAS has been demonstrated in the endocrine pancreas [10,18], we investigated the activation of NAD(P)H oxidase and expression of its components ($p47^{PHOX}$ and $gp91^{PHOX}$) by ANGII stimuli in rat pancreatic isolated islets.

2. Materials and methods

2.1. Reagents

The reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were obtained from Bio-Rad (Richmond, CA, USA). Tris, EDTA, aprotinin, PMSF, dithiothreitol (DTT), Triton X-100, Tween 20, glycerol, angiotensin II, diphenylene iodonium (DPI) and collagenase were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroethidine (HEt) was obtained from Molecular Probes (Eugene, OR, USA). The $p47^{PHOX}$ and $gp91^{PHOX}$ antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-phosphoserine was from Chemicon International (Temecula, CA, USA). The enhanced chemiluminescence reagent kit, ECL, was from GE Healthcare (Buckinghamshire, UK). The AT1 receptor antagonist, losartan was kindly provided by Dr Lisete C. Michelini from University of Sao Paulo, Brazil.

2.2. Animals

The experiments were performed following the guidelines of the Animal Research Ethics Committee of the Federal University of Sao Paulo — UNIFESP. The rats were kept in groups of five at 23 °C in a room with a light/dark cycle of 12/12 h (lights on at 07:00 h). Each set

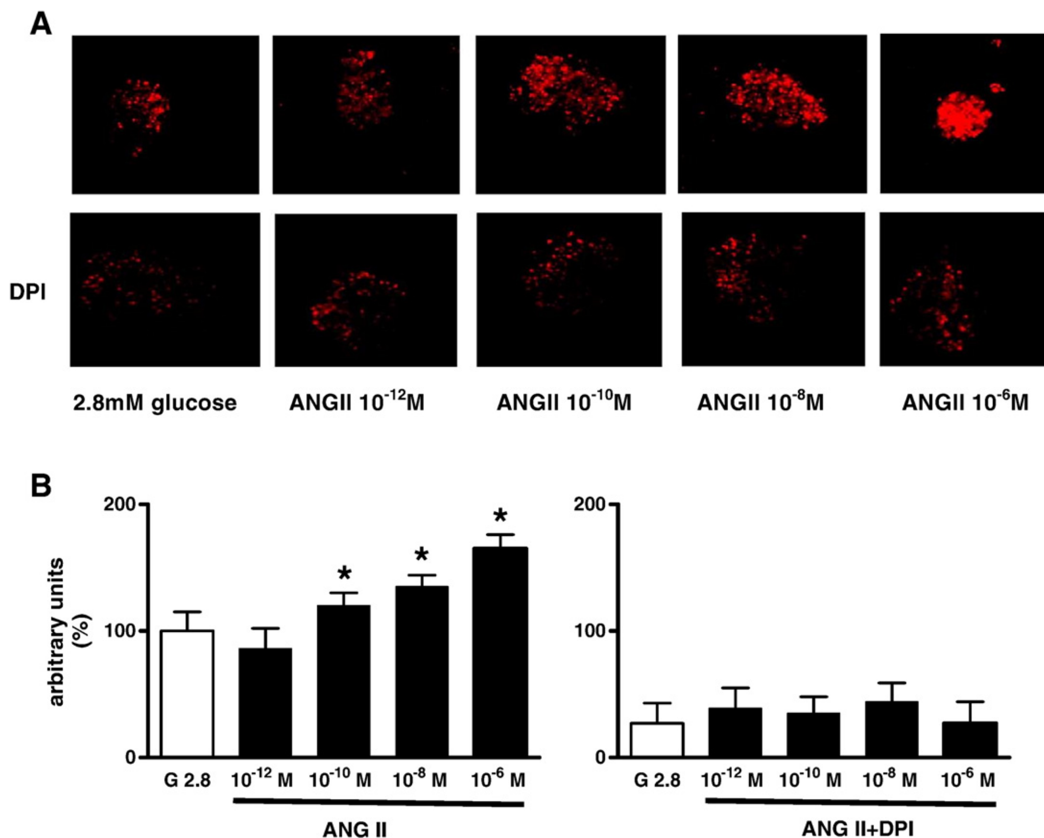

Fig. 1. Involvement of NADP(H) oxidase in ANGII-induced on superoxide production in isolated rat pancreatic islets. Pancreatic islets were incubated at 37 °C in the Krebs–Henseleit/KH buffer (139 mM Na^+, 5 mM K^+, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 124 mM Cl^-, 24 mM HCO_3^-) in the presence of ANG II in different concentrations —10^{-12} M , 10^{-10} M , 10^{-8} M , 10^{-6} M (A—upper panel) and DPI (10 µm/L), the NAD(P)H oxidase inhibitor (B—lower panel). Glucose 2.8 mmol/l was the control group. Superoxide production was determined by HEt assay (fluorescence was excited at 547 nm). Islets were visualized in a confocal microscope (Carl Zeiss, Morris Plains, NJ, USA) and 3 µm laser scanning sections were analysed (A); Representative bars are shown in B. Results were expressed as means±SE $*p<0.05$ as compared to 2.8 mM glucose.

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