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ORIGINAL PAPER

In hyperthyroid rats octylguanidine protects the heart from reperfusion damage

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Abstract Hyperthyroidism sensitizes the heart for reperfusion injury. As known, mitochondrial permeability transition underlies reperfusion heart damage. This study was undertaken to explore the protective effect of octylguanidine (OG), an inhibitor of permeability transition, on hearts from hyperthyroid rats subjected to ischemia/reperfusion. Hyperthyroidism was induced by a daily injection of 2 mg T3/kg body weight for 5 days. OG was injected at a dose of 5 mg/kg body weight. It was found that the amine protects against reperfusion-induced permeability transition, i.e., mitochondria from hyperthyroid rats, treated with OG, retained accumulated Ca^{2+}, similarly to control mitochondria. OG maintained post reperfusion cardiac frequency in hyperthyroid rats at 429 ± 16 in comparison to control and T3 treated rats (70 ± 12 and 71 ± 2, respectively). We also found that OG diminished the post reperfusion accumulation of IFNγ from 34.3 ± 2.5 to 18.7 ± 1.35, IL-6 from 38.5 ± 4.5 to 15.1 ± 0.12, IL-1 from 16.78 ± 0.73 to 12.19 ± 1.54, and TNFα from 45.05 ± 3.14 to 29.85 ± 4.3 (pg/50 μg myocardial tissue). It is concluded that OG inhibits the hypersensitivity of the hyperthyroid myocardium to undergo reperfusion damage due to its inhibitory action on the permeability transition pore.

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Introduction

Hyperthyroidism is frequently associated with hyperdynamic circulation with increased cardiac output, heart rate, pulse pressure, and high blood pressure, as well as a decrease in vascular peripheral resistance [1, 2]. Hyperthyroidism is also associated with an improved metabolic state characterized by an increased mitochondrial function due to a high induction of gene expression of respiratory proteins by T3 [3]. The above is associated with overproduction of reactive oxygen-derived species [4], which induces significant tachycardia [5, 6]. On the other hand, a hyperthyroid state makes the heart greatly susceptible to undergo severe tissue damage after reperfusion following an ischemic period [7]. Abrupt reoxygenation by the implant of intracoronary stents or a coronary by-pass has been successfully used to allow blood reperfusion. However, it causes significant cell injury due to the increased production of oxygen-derived reactive species, formed through different mechanisms [8, 9]. Further, the resulting cellular Ca^{2+} accumulation contributes significantly to the myocardial insult [10, 11]. The increase in cytosolic Ca^{2+}, at the end, results in its overload within mitochondria, and contributes to leakage of the inner membrane. At present, there is a consensus that the switch of permeability from specific to nonspecific, due to the opening of a transmembrane pore, plays a central role in the heart injury that follows reperfusion [12]. Recently, Wajima et al. [13] showed that inhibition of the mitochondrial K/ATP channel reduced myocardial infarct size after ischemia/reperfusion.

Recently, we found that hypothyroidism renders liver mitochondria resistant to undergo permeability transition [14]. In agreement, we further demonstrated that hypothyroidism provides resistance to the myocardium against reperfusion injury [15]. To explain these findings we proposed that the lack of cardiolipin in the lipid phase of the inner mitochondrial membrane restrains the shift of adenine nucleotide translocase (ANT) to the nonspecific transmembrane pore. It should be noted that T3 modulates the activity of mitochondrial cardiolipin synthase [16], the genomic expression of ANT [17], and the mitochondrial calcium uniporter [18]. Thus, under hyperthyroid conditions, mitochondria are more susceptible to suffer permeability transition [19]. From the above, it is understandable why hyperthyroidism exacerbates the pathogenesis of myocardial reperfusion injury [20, 21]. Moreover, L-thyroxin stimulates Ca^{2+}-induced membrane leakage [22] and oxidative stress through an increase in the rate of superoxide radical generation [23].

It is known that during myocardial reperfusion damage, after a period of ischemia, opening of the nonspecific pore occurs [12, 24]. Although the nature of the components of the pore remains controversial, it seems that ANT regulates the open/closed cycles. Molecules that interact directly with this carrier, i.e., carboxyatractyloside and the enzyme cyclophilin D induce pore opening, whereas agents like cyclosporin A, interacting with cyclophilin D, close the pore [25]. In a previous work, we demonstrated that octylguanidine (OG) prevents mitochondrial membrane leakage, i.e., permeability transition, triggered by Ca^{2+} overload [26]. Besides, the ability of OG to avoid heart damage promoted by ischemia/reperfusion [27] was also documented. The mechanism by which OG blocks the pore and, in consequence, protects from reperfusion damage, could be explained as follows: the positive charge of the guanide group might interact with negative charges of amino acid residues of the translocase; certainly, the carrier possesses 15 glutamate and 6 aspartate residues [28], these residues may form a negative pocket for the guanidinium group, whereas the alkyl chain penetrates into the hydrophobic milieu of the inner membrane, fixing the carrier in the closed conformation. Based on the aforementioned, this work was undertaken with the purpose of exploring the protective role of OG on hyperthyroidism-induced myocardial damage.

Results

Cardiac frequency after reperfusion

Hyperthyroidism, associated with reperfusion-induced myocardium calcium overload, induces an increase of cardiac frequency and arrhythmias. Figure 1 shows ECG tracings from control (Ctrl), hyperthyroid (T3), and T3 plus OG-treated rats. Panel A depicts the electric profile of

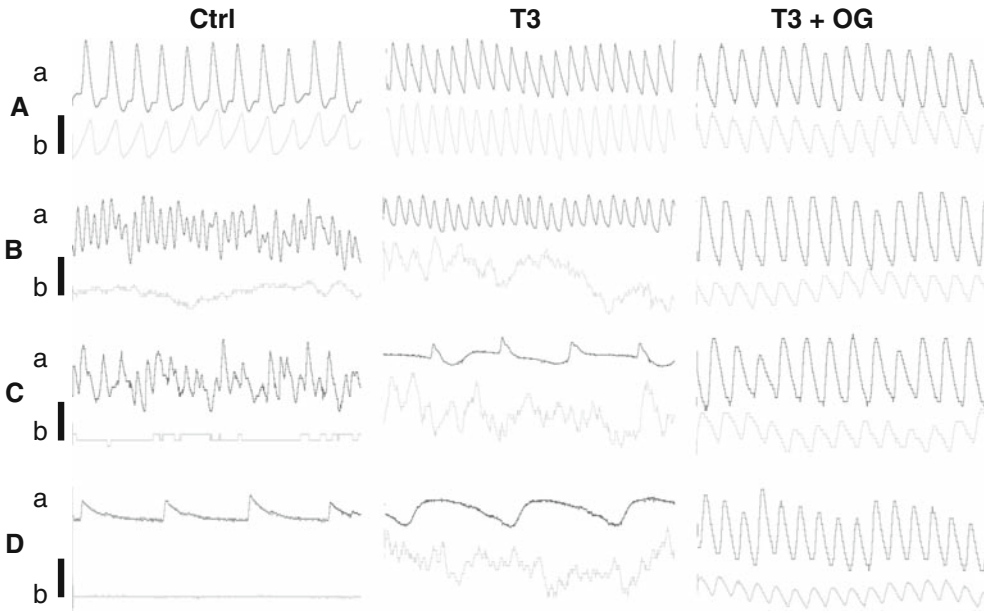

Fig. 1 Electrocardiogram and blood pressure tracings of hearts from control, hyperthyroid, and hyperthyroid plus OG-treated rats. Panel A shows ECG tracings before the ischemic period. Panel B illustrates ECG tracings during 5 min of ischemia. Panel C indicates the ECG tracing during 5 min of reperfusion, and Panel D shows ECG tracings obtained during the 20-min reperfusion period. ECG traces are indicated with a in each panel. Lower traces, indicated with b in each panel correspond to blood pressure. The bars correspond to 100 mmHg. The traces are representative of six separate experiments

hearts before being subjected to ischemia. As observed, the hearts from control, T3, and T3 plus OG-treated rats were in a sinus rhythm; in addition, blood pressure remained unchanged. Panel B shows ECG tracings from hearts undergoing an ischemic period of 5 min. It is clearly observed that hearts from control, as well as T3-treated, rats present ventricular tachycardia. Such increased cardiac frequency was absent in hearts from T3 plus OG-treated rats. When blood-reflow was established, during 5 min (Panel C), the incidence of ventricular tachycardia was increased in euthyroid rat hearts. Interestingly, hearts from T3-treated rats showed a remarkable bradycardia. It should be noted that the slow resting hearth rate was not present in hearts from T3-OG treated rats. As indicated in Panel D, after 20-min reperfusion, bradycardia was more apparent in hearts from control and hyperthyroid rats. A different picture is revealed by the ECG tracings (a) from T3-OG rats; as observed, abnormal beats were almost absent and the hearts remained in sinus rhythm. Blood pressure, lower traces (b) in their respective panels, was almost absent in control and hyperthyroid rats. In contrast, in T3 plus OG-treated rats, the magnitude of this variable was maintained within normal values. A statistical analysis of the results obtained after 5 min ischemia followed by 5 and 20 min of reperfusion is shown in Table 1. As indicated, at 5 min of reperfusion, T3 plus OG-treated rats maintained their heart rates at values similar to those found in T3 rats (463 ± 20 and 393 ± 177, respectively). However, after 20-min reperfusion, OG-treatment was efficient to preserve the heart rate at a normal value of 429 ± 16, in contrast to the value in T3-treated rats, which was 71 ± 2. Also, OG was efficient to sustain blood pressure after 20-min reperfusion. As illustrated, this parameter was maintained at 106 ± 20 mmHg, in comparison to that observed in T3-treated rats: 3 mmHg.

Table 1 Analysis of cardiac frequency and blood pressure of control and T3 or T3-OG treated rats

	Control	T3	T3 + OG
Basal			
Heart rate (beats/min)	306 ± 19	562 ± 8**	476 ± 34**
Blood pressure (mmHg)	110 ± 4	145 ± 7**	104 ± 11**†
5-min ischemia			
Heart rate (beats/min)	250 ± 45	590 ± 41**	495 ± 28**
Blood pressure (mmHg)	60 ± 6	64 ± 13	113 ± 4**†
5-min reperfusion			
Heart rate (beats/min)	736 ± 66	393 ± 177**	463 ± 20**
Blood pressure (mmHg)	30 ± 5	10 ± 4†	93 ± 10**†
20-min reperfusion			
Heart rate (beats/min)	70 ± 12	71 ± 2	429 ± 16**†
Blood pressure (mmHg)	8	3	106 ± 20**†

The values represent the average ± S.D. of six separate experiments. Statistical analysis by non-parametric one-way ANOVA with either Student-Newman-Keuls or Dunet's test using Prism 4.0 software

** $P < 0.001$ respect control value, † $P < 0.001$ respect T3 value. Experimental conditions were as described under Methods

Mitochondrial calcium movements

T3 treatment results in an abrupt increase in mitochondrial nonspecific permeability opening of the permeability transition pore and underlies the reperfusion heart damage. Permeability transition is characterized by the release of mitochondrial matrix content, i.e., Ca^{2+}. Thus, the experiment shown in Fig. 2 was performed to assess the protective effect of OG on membrane leakage. This figure shows Ca^{2+} release from mitochondria isolated from hyperthyroid heart rats, subjected to 5-min ischemia followed by 20-min reperfusion, treated or not with OG. As observed, in trace a, T3-treated mitochondria were unable

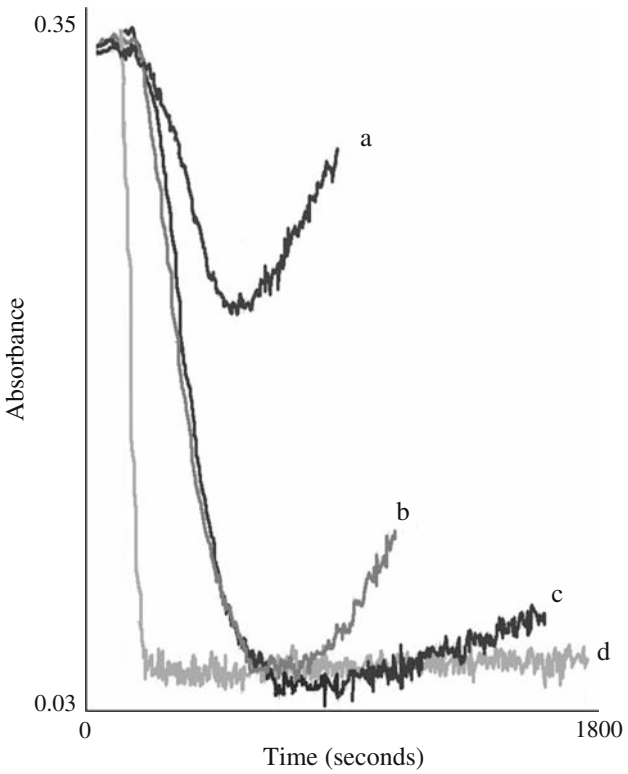

Fig. 2 Effect of OG on calcium retention by heart mitochondria isolated from hyperthyroid rats subjected to ischemia reperfusion. Mitochondrial protein (2 mg) was incubated in 3 ml of a medium containing 125 mM KCl, 10 mM succinate, 10 mM HEPES, 3 mM phosphate, 100 μM ADP, 50 μM $CaCl_2$, 5 μg rotenone, 2 μg oligomycin, and 50 μM Arsenazo III. Trace a indicates mitochondria from hyperthyroid mitochondria subjected to ischemia/reperfusion. Trace b shows euthyroid mitochondria from ischemic-reperfused hearts. Trace c illustrates the behavior of hyperthyroid mitochondria-treated with OG from hearts subjected to ischemia–reperfusion. Trace d shows Ca^{2+} retention by control mitochondria. The traces are representative of six separate experiments. Temperature 25°C

to retain Ca^{2+} as a consequence of pore opening. Trace b shows that euthyroid mitochondria, isolated from ischemic/reperfused hearts, behave in a similar way. However, the opposite occurred in mitochondria from hyperthyroid rats treated with OG (trace c); as shown, Ca^{2+} was maintained accumulated in the matrix, indicating that the pore remained closed. Trace d shows calcium retention by control mitochondria.

Mitochondrial oxygen consumption

The experiment shown in Table 2 was performed to explore further the protective effect of OG on the damage of hyperthyroid mitochondria, isolated from the left ventricle, and subjected to ischemia/reperfusion, to achieve oxidative phosphorylation. As shown, respiratory control from euthyroid mitochondria that were not subjected to ischemia/reperfusion attained a respiratory control value of 5.76 ± 0.06; however, this value diminished to 2.42 ± 0.4 after 5-min ischemia and 5-min reperfusion. After 5-min ischemia/20-min reperfusion, respiratory control almost disappeared, decreasing to 1.62 ± 0.4. It is also shown that the respiratory control value in hyperthyroid mitochondria, not subjected to ischemia reperfusion, was lower than in control mitochondria, it was 3.13 ± 0.32. After 5-min ischemia/5-min reperfusion, hyperthyroid mitochondria showed a respiratory control of 1.89 ± 0.2. When subjected to 5-min ischemia/20-min reperfusion, hyperthyroid mitochondria lost almost completely the ability to build up a respiratory control, the value was 1.0 ± 0.2. Remarkably, mitochondria from T3 plus OG-treated rats increased the respiratory control values, in comparison to those shown by OG-untreated rats, attaining values of 2.73 ± 0.09 and 3.97 ± 0.1, after 5-min ischemia/5-min reperfusion and after 5-min ischemia/20-min reperfusion, respectively. Here, mitochondrial respiratory control is defined as the ratio between the rate of oxygen consumption (nAtg O_2/min/mg protein) after the addition of ADP and the rate of oxygen consumption without ADP.

Table 2 Respiratory control of mitochondria isolated from hearts of control, hyperthyroid-untreated, and hyperthyroid OG-treated rats subjected to 5-min ischemia and 5- or 20-min reperfusion

	Basal	5-min ischemia/ 5-min reperfusion	5-min ischemia/ 20-min reperfusion
Control	5.76 ± 0.06	2.42 ± 0.4	1.62 ± 0.4
T_3	3.13 ± 0.32**	1.89 ± 0.2	1.00 ± 0.2
T_3 + OG	3.72 ± 0.80	2.73 ± 0.09	3.97 ± 0.1**†

Mitochondria (0.5 mg protein) were incubated in 1.5 ml of a medium similar to that described for Fig. 1, except that Arsenazo III and Ca^{2+} were not added. Temperature, 25°C. The values are expressed as mean ± S.D. of six different experiments. Statistical analysis by non-parametric one-way ANOVA was done with either Student-Newman-Keuls or Dunet's test using Prism 4.0 software

** $P < 0.001$ respect control group under the same conditions, † $P < 0.001$ respect T3 value

Cytokines measurements

Both, overproduction of thyroid hormones and reperfusion injury, raise serum interleukins. The experiment of Fig. 3 was aimed at exploring the effect of OG on the release of IFNγ, IL-6, IL-1, and TNFα, after reperfusion in T3-treated heart rats. As observed, after 20-min reperfusion, OG partially avoided the release of these cytokines: IFNγ was diminished from 34.3 ± 2.5 to 18.7 ± 1.35; IL-1 from 16.78 ± 0.73 to 12.19 ± 1.54; IL-6 from 38.5 ± 4.5 to 15.1 ± 0.12, and TNFα from 45.05 ± 3.14 to 29.85 ± 4.3, although the effect of OG was more apparent in diminishing the increased amount of IFNγ and IL-6.

Histological studies

Figure 4, Panel A and D, shows the histological studies of myocardial tissue from T3 rats not subjected to ischemia/reperfusion and untreated or treated with OG, respectively. Panel B and C show the image of the myocardium from T3 rats subjected to ischemia, and 5- or 20-min reperfusion, respectively, not treated with OG. Panel E and F illustrate the myocardial tissue from T3 rats treated with OG, after 5- or 20-min reperfusion. The image from OG-treated rats shows that the architecture of the tissue was better preserved; a better striation of myocardial fibers and almost no edema are observed.

Discussion

Among the several manifestations of hyperthyroidism are alterations in the cardiovascular system. The signs and symptoms comprise increased blood pressure, atrial and ventricular extra-systoles, atrial fibrillation, and ventricular repolarization abnormalities [20]. Further, hyperthyroidism sensitizes the rat heart to reperfusion injury, characterized by severe arrhythmias and tissue injury [21]. At the subcellular level, L-thyroxin stimulates mitochondrial Ca^{2+} overload, inducing inner membrane leakage [19, 22]. The latter brings about the opening of the nonspecific transmembrane pore, a process that leads to mitochondrial dysfunction, and underlies the pathogenesis of heart reperfusion damage [11, 29, 30]. From the above, it can be inferred that protection of mitochondria against Ca^{2+}-dependent permeabilization results in resistance to reperfusion-induced damage. To this regard, we demonstrated previously that OG protects mitochondria from

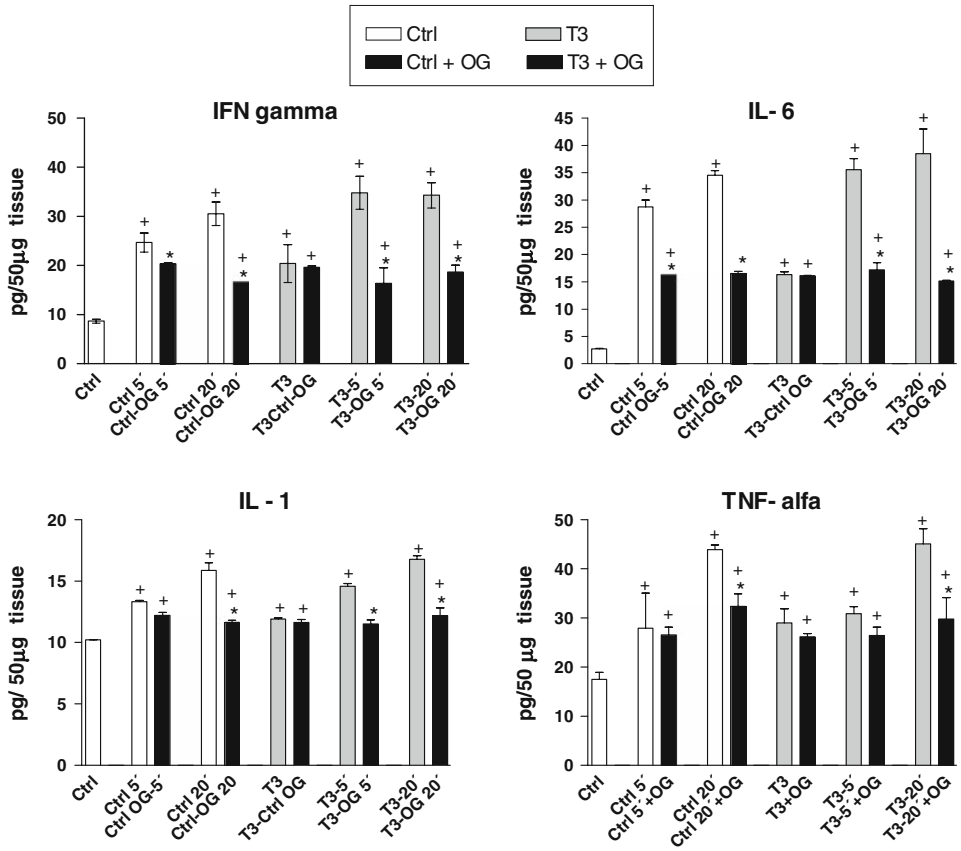

Fig. 3 Effect of octylguanidine on the release of cytokines. Left ventricular myocardium, frozen and ground, was homogenized in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, and 5 mM EGTA containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail. Lysates were centrifuged at 10,000*g*, and protein was determined. Cytokines were determined by using specific antibodies through ELISA. The values represent the average of six separate experiments ± S.D. Statistical analysis was done by non-parametric one-way ANOVA with either Student-Newman-Keuls or Dunet's test using Prism 4.0 software. $^{*}P < 0.001$ respect the same group without OG, $^{+}P < 0.001$ respect to the control group

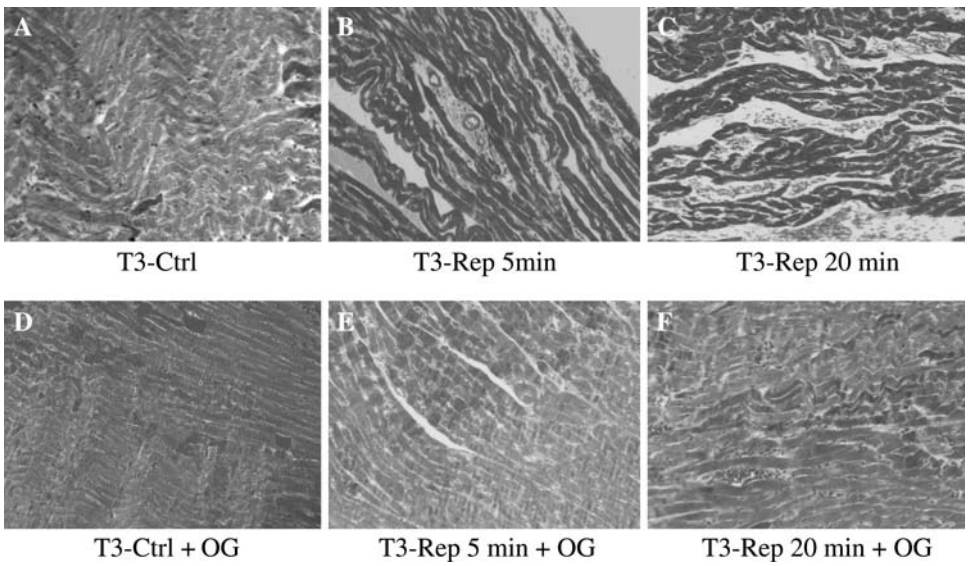

Fig. 4 Histological image of cardiac tissue after reperfusion. Panel A and D show heart images from T3-treated rats without reperfusion, treated and untreated with OG. B and C show images of the myocardium from T3 rats subjected to 5- or 20-min reperfusion, respectively, untreated with OG. Panels E and F illustrate images of the myocardium from T3 rats subjected to 5-min ischemia/5-min reperfusion and 5-min ischemia/20-min reperfusion, treated with OG

permeability transition as induced by carboxyatractyloside, through blocking ANT [26]. We also showed that OG maintains the myocardium in a good shape, protecting it against the damage induced by ischemia/reperfusion [27].

The results in this work indicate that OG inhibited reperfusion arrhythmias and reduced the incidence of ventricular tachycardia in hearts isolated from hyperthyroid rats. There are reports indicating that hyperthyroidism amplifies the susceptibility of mitochondria to undergo permeability transition [19], as well as the susceptibility of the myocardium to oxidative stress and reperfusion damage [23]. As was also shown, OG inhibited permeability transition in hyperthyroid mitochondria. This assumption emerges from the fact that mitochondria isolated from T3

plus OG-treated rats were able to retain accumulated Ca^{2+}, in comparison to those isolated from OG-untreated hyperthyroid mitochondria, and preserved the ability to synthesize ATP, as can be inferred from the conservation of the respiratory control within acceptable values. The latter can be explained considering that OG inhibits membrane leakage; thus allowing the inner membrane to build up a transmembrane proton gradient, required for oxidative phosphorylation.

There are experimental models indicating a close association between cellular Ca^{2+} overload and alterations in the heart rhythm [11]. Intracellular calcium increases during myocardial ischemia [10, 11]; however, when blood flow is restored, in the reperfusion period, a rapid and excessive uptake of Ca^{2+} can occur with adverse electrophysiological effects, including the development of calcium-dependent arrhythmias. García-Rivas et al. [11] demonstrated that Ru360, a specific mitochondrial calcium uptake inhibitor, improves cardiac post ischemic functional recovery. Massive Ca^{2+} load is a consequence of the lack of ATP, and implies Ca^{2+}-dependent ATPase dysfunctions; hence, a loss in the homeostasis of intracellular Ca^{2+}. In this context, OG, by preserving mitochondrial selective permeability, may improve oxidative phosphorylation, diminishing cytosolic Ca^{2+} accumulation and, in consequence, diminishing electric and rhythm alterations in the myocardium. As a consequence of the above, blood pressure is restored to normal values. To this regard, reperfusion following ischemia induces a progressive decline in cardiovascular functions, evidenced by a decrease in mean arterial blood pressure, cardiac output, and ejection fraction [31]. The harmful effects on heart failure and hypotension are magnified in thyrotoxicosis [32]. Noticeable, as demonstrated by the results shown in Fig. 1, OG preserved the values of heart rate and blood pressure within control values, independently from the length of the reperfusion period.

The release of cytokines is an important factor in the acute inflammatory process following myocardial ischemia–reperfusion [33, 34]. Further, thyrotoxicosis is also associated with a rise in serum interleukins [35]. Our results show that IL1β, IL-6, TNFα, and IFNγ were increased in heart tissue of hyperthyroid rats subjected to ischemia–reperfusion. Interestingly, OG-treatment diminished the values of these cytokines almost to those found in the hearts from control rats. In addition, we found that OG inhibited cytokines release in cultured lymphocytes (data not shown). The mechanism by which OG has an immunosuppressive-like effect is an open question. However, several reports indicate that the polyamine spermine inhibits proinflamatory cytokine synthesis, among them TNFα and IL-1. The authors discuss that the inhibition is post transcriptional, through a pathway different from glucocorticoids [36]. A similar mechanism could be ascribed to OG. At this moment, we are performing appropriate experiments to offer a plausible explanation.

Materials and methods

Rat hyperthyroidism

Hyperthyroidism was induced in male Wistar rats, weighing between 300 and 350 g, by a daily i.p. injection of 2 mg 3,5,3′-thriiodothyronine (T3)/kg body weight for 5 days. This investigation was carried out according to the procedures published by NIH for laboratory animals, and following the approved procedures by the Bioetics Commission of the Instituto Nacional de Cardiología de México.

Octylguanidine administration

Octylguanidine was a generous gift of Dr. Antonio Peña from the Instituto de Fisiología Celular, UNAM, who synthesized it according to Phillips and Clarke [37]. The mass and purity of the reagent was assessed by Dr. Roberto Arreguín from the Instituto de Química, UNAM, by using two methods, mass spectrometry and high performance liquid chromatography, indicating that the purity attained was 90% and 98%, respectively, with a molecular weight of 171 Da, which agrees closely with its molecular composition. In agreement with previous work [27], the amine was administered at a dose of 5 mg/kg body weight, injected through the femoral vein 10 min before starting the ischemic period.

Heart reperfusion

Heart damage by ischemia/reperfusion in control and hyperthyroid rats was achieved as follows: the rats were anesthetized with sodium pentobarbital (55 mg/kg, i.p.) and maintained under assisted respiration through a thoracotomy. One lead-II surface electrocardiograph was used to monitor heart rate; blood pressure was measured with a pressure transducer attached to a femoral cannula. The chest was opened by thoracotomy; the left coronary artery was ligated near its origin by an intramural 6-0 silk loop. Occlusion of the artery was performed by passing a short tube over the vessel and clamping it firmly. The ischemic period lasted 5 min. This time was selected in agreement with previous reports [15, 27, 38]. Nevertheless, to get a better insight about the myocardial damage, experiments with 20 min of reperfusion were also performed. Reperfusion was started by removing the clamp, and lasted 5 min. Those heart beats that were able to raise blood pressure were considered normal. T3 was determined by a

chemiluminescence assay in rat blood serum, the values, expressed as mean of six different samples ± S.D, were 267 ± 36.47 ng/dl for control rats and 3057 ± 174.8 ng/dl for T3-treated rats, $P < 0.0001$.

Determination of cytokines

At the end of the experiment, samples of the left ventricular wall were obtained to estimate the amount of released IL1, IL6, IFNγ, and TNFα, according to Palmieri et al. [39]. The values of these myocardial cytokines were analyzed through a sandwich ELISA method [40].

Mitochondrial preparation

Mitochondria from the left ventricle were prepared after homogenization of the tissue of the left ventricle in 250 mM sucrose-1 mM EDTA adjusted to pH 7.3, and following the standard centrifugation procedure. Protein was determined according to the Lowry method [41]. Ca^{2+} uptake was followed spectrophotometrically at 675–685 nm using the indicator Arsenazo III, by incubating mitochondria in a medium described in the respective figure.

Mitochondrial respiration

Oxygen consumption was analyzed polarographically using a Clark-type electrode, by adding 0.5 mg of mitochondrial protein to 1.5 ml of medium containing 125 mM KCl, 10 mM succinate, 3 mM phosphate, 10 mM HEPES, pH 7.3, and 5 μg rotenone. Oxygen consumption was stimulated after the addition of 100 μM ADP.

Microscopy studies

At the end of the experiment, samples from the ventricular free wall were obtained for histological studies. The tissue was fixed in 10% formol-buffer and sliced at 4 μm thickness, Mason dye was used to visualize the fibers.

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