

Research Article

Glucagon Effects on Ischemic Vasodilatation in the Isolated Rat Heart

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The myocardial reperfusion following ischemia leads to the ischemic vasodilation by affecting the release of various vasoactive substances, such as free radicals, NO, and histamine. In addition, some evidences suggest that glucagon itself may alter the release of those substances. In this study, we investigated the ischemic vasodilation of the isolated rat heart, as well as the concentrations of NO, TBARS, and histamine in the coronary venous effluent either in the presence or in the absence of glucagon. Our results showed that in the presence of glucagon, there was a faster restoration of coronary perfusion pressure during ischemic vasodilation compared to the absence of glucagon (124 ± 5.6 versus 81 ± 5.2 s) with no apparent changes in TBARS concentration. The glucagon's administration leads to the decreased release of histamine by approximately 35%. Biphasic release of NO in the presence of glucagon initially showed augmentation by 60%, followed by the significant attenuation of 45%.

1. Introduction

Although the myocardial reperfusion following ischemia can produce the salvage of the ischemic tissue, it may also contribute not only to the endothelial dysfunction and the myocardial cellular injury, but it also may change the release of the biologically active substances, such as the endothelium-derived relaxing factor (NO) [1]. Even though NO obtained from L-arginine is a free radical *per se*, it also can quench other free radicals, including the superoxide radicals and it thereby protects both the endothelial cells and the cardiac myocytes [2, 3]. In addition, NO may stabilize mast cells and inhibit the release of histamine from the both isolated and the resident cardiac mast cells, thus influencing the postischemic myocardial injury [4–6]. Thus, low doses of NO appear to play a key protective role from the myocardial ischemia-reperfusion injury. However, high concentrations of NO exert highly toxic and harmful effect during the ischemia-reperfusion injury [3, 7–9]. Beside the fact that NO may stabilize the cardiac mast cells and it consequently decreases the histamine release [4, 5, 10], earlier results [11] showed a positive feedback relationship between NO

and histamine in the regulation of both coronary ischemic vasodilation and the coronary autoregulation.

In our previous work, we evaluated both the dynamic response and the biomechanical properties of the isolated blood vessels in the presence of NO precursor, L-arginine [12]. Our results demonstrated that some relaxation, such as the increase in the diameter of the blood vessel, was evident in the presence of L-arginine. At the same time, the development of the pressure was significantly faster compared to the group without the L-arginine. The analysis of the biomechanical parameters, such as the stress-strain and the shear stress, suggested that the isolated blood vessel became more rigid in the presence of L-arginine.

The myocardial ischemia and the reperfusion have also been suggested to increase the histamine production [13]. The histamine not only exists (to a great extent) within the mast cells and the basophilic leukocytes, but it also could be released by the endothelial cells, the aggregating platelets, lymphocytes and monocytes/macrophages [14, 15]. High concentrations of the histamine are present in the cardiac tissue of most animal species, including humans [13] provoking various cellular functions via the stimulation of

four different G-protein-coupled receptors (H_1 , H_2 , H_3 , and H_4). The stimulation of the H_2 receptors by the histamine induces both positive inotropic and positive chronotropic effects [16, 17]. It has been shown that the blockage of the histamine H_2 receptors improved the anaerobic metabolism of the myocardium in the ischemic heart disease that has been associated with the reduced myocardial oxygen consumption demonstrating the highly beneficial effect against the ischemia-reperfusion injury. Moreover, the antagonists to the H_2 -histamine receptors in the mast cells of the heart may reduce the activity of the mast cells, thereby; they may reduce the release of the mast cell's mediators [13, 18, 19]. Even though the blockage of the histamine H_2 receptors limited infarct size, the inhibition of the histamine H_1 receptors did not demonstrate this beneficial effect [13]. In the heart, the stimulation of the H_1 receptors with histamine impairs the atrioventricular (AV) conduction, thus contributing to the development of the ventricular fibrillation in the myocardial ischemia-reperfusion injury [20, 21]. Furthermore, the mast cells play a role in the generation of the reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide, that are responsible for the further increased release of the histamine from the mast cells and vice versa [6]. The histamine effects on the coronary arteries are the result of the multiple actions of this molecule on both smooth muscle cells and the endothelium. Some reports showed different effects of the histamine including both the relaxation and the constriction of the coronary vessels depending on species, dose of the histamine, the diameter of the blood vessels and the initial vessel's tone, as well as the relative location within the coronary circulation [22].

The glucagon is an endogenous polypeptide hormone that exerts both positive inotropic and the chronotropic effects on the myocardial tissue [23, 24]. The investigations of the glucagon influence on the transport of the radiolabeled histamine in the isolated guinea pig heart demonstrated the elevation of the histamine transport in the presence of glucagon [25, 26]. Previous findings showed that the cardiac anaphylactic crisis was markedly reduced in the presence of glucagon. Furthermore, these data suggested that the antiarrhythmic action of glucagon during the cardiac anaphylaxis involved the inhibition of histamine release, the vasodilation of the coronary vessels, the increase in the automaticity of the sinoatrial node, and the enhancement of the atrioventricular conduction velocity [27]. Moreover, it has been demonstrated that the glucagon influenced the accumulation of histamine in the vesicles of enterochromaffin-like cells [28]; however, until now there have not yet been reported data explaining the glucagon-induced stimulation of the histamine storage in the heart cells.

We postulated that glucagon can affect the changes of the coronary perfusion pressure during the ischemia-reperfusion, by altering the release of NO and/or histamine in the heart. Therefore, in the present study, we investigated the changes in the coronary perfusion pressure during the ischemia-reperfusion period, as well as the concentrations of nitric oxide, thiobarbituric acid reactive substances (TBARS), and histamine either in the presence or in the absence of glucagon in the coronary venous effluent of the

isolated rat heart. Here, we applied the experimental model and originally developed mathematical procedures (12) to describe the dynamic response of the coronary blood vessels.

2. Material and Methods

All experiments were performed according to EU (86/609/EEC) and the Local ethical guidelines. Wister rats of both sexes (200–250 g body weight) were killed by the cervical dislocation. The rat hearts were rapidly isolated and retrogradely perused via the aorta, according to the Langendorff's technique at a constant flow (7–8 mL/min/g wt) of the perfusion buffer containing (in mM): NaCl 118.1, KCl 4.7, $MgSO_4$ 1.66, $NaHCO_3$ 24.88, KH_2PO_4 1.18, glucose 5.5, sodium pyruvate 20, $CaCl_2 \times 2H_2O$ 2.52. The perfusate was continuously bubbled with 95% O_2 and 5% CO_2 , with the pH adjusted to 7.4 at 37°C. The coronary perfusion pressure was measured by a pressure transducer (Elunit, Yugoslavia) and recorded by a Kipp & Zonen chart recorder (UK). The electrical pacing of the isolated hearts (3 V, 2 ms, 300 bpm) was performed with the electrodes placed on the right auricles near SA node.

After the equilibration period (20 minutes), the ischemic vasodilation was induced by the interruption of coronary inflow (global ischemia) for 60 seconds. Two to three bouts of the ischemic vasodilation were induced in each heart, both without (the control group, $n = 10$) and with glucagon solution (the test group, $n = 10$) approximately 15–20 minutes between the interruptions of the coronary inflow. Glucagon (Sigma, USA) was added in the physiological solution in the final concentration of 400 nM, at least 2 minutes before the occlusion, and the addition continued during the reperfusion, until 2 minutes after the reestablishment of the coronary pressure to the preischemic level. The samples of the venous effluent were continuously collected during the reperfusion period and prepared for further analysis: the determination of the histamine concentration, and the levels of NO and TBARS.

The histamine concentration was measured using Shore's fluorometric method [29].

After the coronary occlusion, the coronary pressure decreased near to zero. Following the period of ischemia, the coronary flow was reestablished (the reperfusion period) and the changes in the coronary perfusion pressure (CPP) versus time were recorded.

The experimentally recorded dependence of CPP on time during the reperfusion period was fitted using an exponential mathematical function:

$$y = b_1 \left(1 - e^{-b_2 x}\right), \quad (1)$$

where y was the CPP (in mmHg), x was the time (in s), and b_1 and b_2 were the coefficients of this relationship:

- (i) b_1 has units of pressure, and
- (ii) b_2 has units of time^{-1} .

This function is shown in the Figure 1. as the CPP versus time curve. The constant b_1 represents the maximum

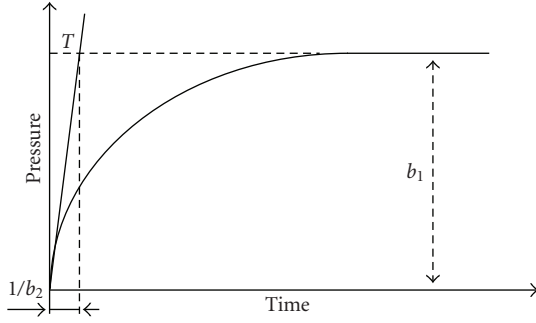


FIGURE 1: Exponential curve $y = b_1(1 - e^{-b_2x})$.

developed pressure, that is, the pressure corresponding to the alternate steady state.

We introduced a dominant time constant (T) as the time value corresponding to the cross section point between the asymptote of the exponential curve and the tangent of the exponential curve at the zero point. This constant results from the following function (1):

$$T = \frac{1}{b_2}. \quad (2)$$

We considered that the alternate steady state was reached for $t = 5T$ ($t = \text{time}$), because in this case, $e^{-t/T} = e^{-5} \approx 0$, and $y \approx b_1$.

Beside the time duration of the CPP restoration (or the flow restoration), the area above the CPP or the flow curve had been commonly used as the parameter for the estimation of CPP or the flow restoration after the ischemic period, by means of the conventional mathematical methods. In this paper, the area above the experimental CPP curve was defined as $eAIV$ (area of ischemic vasodilation).

The area above the fitted curve- $fAIV$ (Figure 2) can be defined by the following function (3):

$$fAIV = \int_0^{\infty} [b_1 - b_1(1 - e^{-b_2x})] \quad (3)$$

and calculated from the following function (4):

$$fAIV = \frac{b_1}{b_2}. \quad (4)$$

2.1. Nitrite Determination. Nitric oxide was assessed as nitrite and quantified by the spectrophotometric method using the *Griess*-reagent. 0.5 mL of the perfusate was precipitated with 200 μL of 30% sulfosalicylic acid, vortexed for 30 minutes and centrifuged at 3000 \times g. The equal volumes of the supernatant and Griess's reagent, containing 1% sulfanilamide in 5% phosphoric acid/0.1% naphthalene ethylenediamine-dihydrochloride were added and incubated for 10 minutes in the dark and read at 543 nmol/l. The nitrite levels were calculated using sodium nitrite as the standard [30].

2.2. Index of Lipid Peroxidation (Thiobarbituric Acid Reactive Substances-TBARS). The degree of lipid peroxidation in the

TABLE 1: Histamine concentrations in the control (without glucagon) and test (in the presence of glucagon) groups taken during preischemic and reperfusion periods.

	Control group	Test group
Histamine concentration in preischemic period (μM)	$4.6 \pm 0.8 \cdot 10^{-8}$	$5.1 \pm 0.6 \cdot 10^{-8}$
Histamine concentration within reperfusion period (μM)	$10.4 \pm 0.9 \cdot 10^{-8*†}$	$6.8 \pm 0.5 \cdot 10^{-8*†}$

Values are represented as mean \pm S.E.M.

(*) Represents significantly different values between control and test groups, $P < .01$; $n = 10$.

(†) Represents significantly different values between histamine concentrations in preischemic and reperfusion periods, $P < .01$; $n = 10$.

coronary venous effluent was estimated by the measurement of thiobarbituric acid reactive substances (TBARS) using 1% TBA (thiobarbituric acid) in 0.05 NaOH incubated with the coronary effluent at 100°C for 15 minutes and read at 530 nm. Krebs-Henseleit solution was used as a blank probe [31].

2.3. Statistical Analysis. Data are presented as mean \pm S.E.M. (standard error of the mean). Statistical analysis was performed by using the multifactorial analysis of the variance for repeated measurements between the subject factors as well as Bonferroni-test. P values less than .05 were considered to be significant. Also, the data were analyzed using Student's t -test, where P value of $< .05$ was considered as statistically significant.

3. Results

To investigate the effect of the glucagon on the concentration of the histamine in the coronary venous effluents, the levels of histamine were measured both in the control and in the test groups either in the absence ($n = 10$) or in the presence ($n = 10$) of the glucagon, respectively. Our results demonstrated no statistically significant differences in the levels of the histamine concentration both in the control and the test groups, as shown in the Table 1. On the other hand, there was a significant increase of the histamine release both in the control (126%) and the test groups (33%) during the reperfusion period compared to the preischemic levels. The increase of the histamine concentrations achieved the maximal values within 30 to 90 s in the control group and within 30 to 60 s in the test group during the reperfusion period. In addition, our results indicated a significant decrease of the histamine release in the presence of glucagon during the reperfusion period compared to the control group.

Furthermore, we measured the levels of NO in the coronary venous effluent, collected both during preischemic and the reperfusion periods, both in the control ($n = 10$) and the test ($n = 10$) groups, as presented in the Figure 3. The

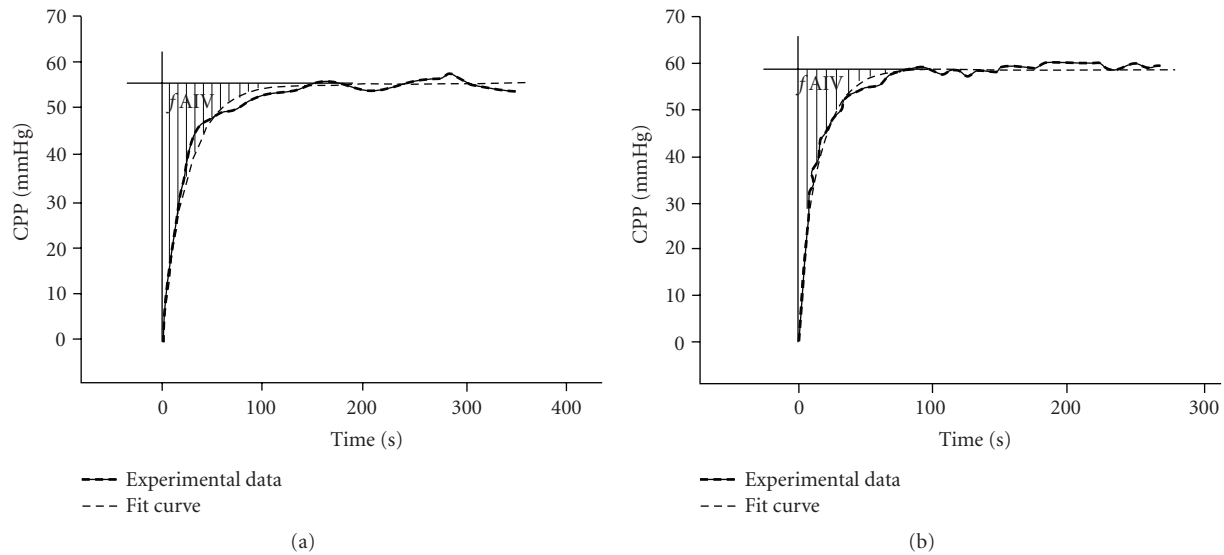


FIGURE 2: Restoration of CPP (original trace) in the absence (a) and in the presence (b) of glucagon (*fAIV* is area of ischemic vasodilation above the fitted CPP curve).

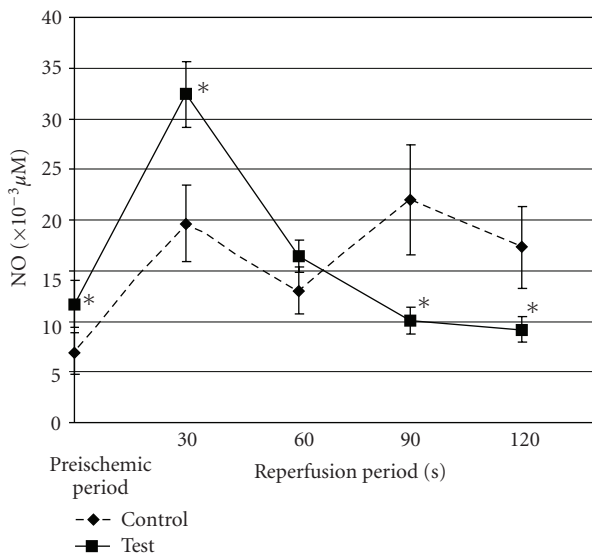


FIGURE 3: NO concentrations in the coronary venous effluent collected during preischemic and reperfusion period in the control and test groups. * represents significantly different values between corresponding values in control and test groups, $P < .01$; $n = 10$.

results showed the increase of NO concentrations in both groups during the reperfusion period. Also, the levels of NO in the test group were significantly elevated (71%) during the preischemic period in comparison to the control group.

The elevated concentrations of NO in the control group collected during the reperfusion period showed two peak values:

- (i) the first peak was evident 30 seconds following the reperfusion period with the increase of 187%, and

- (ii) the second peak showed 90 seconds following the reperfusion period with the increase of 221%.

On the other hand, the NO level in the test group achieved one peak value within first 30 seconds of the reperfusion period. After reaching the peak value, the NO level in the test group gradually decreased until the end of the reperfusion period.

Moreover, within first 30 seconds of the reperfusion period, the NO levels in the test group were initially significantly higher (60%) but eventually reached significantly lower levels (45%) within first 90 seconds of the reperfusion period, compared to the corresponding NO levels in the control group.

Next, we investigated the concentrations of TBARS in the samples collected during both the preischemic and the reperfusion periods, both in the control and in the test groups. Our results showed that the concentrations of TBARS in the samples collected during the reperfusion period were greatly elevated in comparison to the preischemic levels, both in the control group (202.6%, $n = 10$) and in the test group (226.7%, $n = 10$), as shown in the Table 2. The highest TBARS concentrations were achieved within the first 30 seconds of the reperfusion period in both groups. In addition, our results showed no statistically significant differences in the TBARS levels neither in the control nor in the test group both during the preischemic and the reperfusion periods.

Further, we analyzed the experimental CPP-time curves using a method as described in Materials and Methods. The values of $5T$, the b_1 and b_2 coefficients of the fitted experimental data (coefficient of correlation was 0.998 ± 0.002) both in the control ($n = 10$) and in the test ($n = 1$) groups were shown in the Table 3. Our analyzed data indicated significant increase of the coefficient b_2 in the test group compared to the control group. However, the $5T$ value

TABLE 2: TBARS levels in control (without glucagon) and test (in the presence of glucagon) groups taken during preischemic and reperfusion periods.

	Control group	Test group
TBARS concentration in preischemic period (mM)	$6.49 \pm 3.44 \cdot 10^{-6}$	$5.61 \pm 3.41 \cdot 10^{-6}$
TBARS concentration within reperfusion period (mM)	$19.64 \pm 3.17 \cdot 10^{-6}$	$18.33 \pm 2.15 \cdot 10^{-6}$

Values are represented as mean \pm S.E.M ($n = 10$).

TABLE 3: Calculated mathematical parameters (b_1 and b_2) in the control and test groups, and time within which the maximal pressure is developed (taken as $5T$).

Parameter	Control group	Test group
b_1	54.94 ± 4.2	58.28 ± 3.7
b_2	0.04 ± 0.003	$0.062 \pm 0.004^*$
$5T$ (s)	125.4 ± 5.6	$81.7 \pm 5.2^*$

Values are represented as mean \pm S.E.M.

(*) represents significantly different values between control and test group, $P < .01$; $n = 10$.

in the test group was significantly lower in comparison to the value of the control group. Moreover, our results showed no great difference in b_1 coefficients between the control and the test groups of the animals.

When analyzing the $fAIV$ in both the control and the test groups, our results showed that $fAIV$ in the control group was 1373 ± 33 , while in the presence of glucagon this area was significantly lower (940 ± 28). At the same time the values of $eAIV$ both in the control group (1360 ± 29) and the test group (951 ± 30) were not significantly different in comparison to the corresponding $fAIV$ values.

4. Discussion

The restoration of the coronary perfusion pressure during the reperfusion period in our experiments had an exponential form rather than the step function, indicating that the ischemic vasodilation occurred within this period.

In this study, we investigated the ischemic vasodilation of the isolated rat heart, as well as the NO, TBARS, and histamine concentrations in the coronary venous effluent either in the presence or in the absence of glucagon. We used a new approach [12] in the ex vivo experiments considering not only the end points of the vessels response, but also the dynamics of this response. In order to elucidate the mentioned phenomena, we described the dynamic behavior of the coronary blood vessel during the ischemic vasodilation by proposing a mathematical relation where the parameters were evaluated through the relationships of the coronary pressure-time curves with the experimental data. The coronary pressure-time curves were fitted using the simple exponential mathematical function in the way that both b_1 and b_2 coefficients had the physical meaning. In this function, the first coefficient— b_1 —numerically

described the maximal developed coronary pressure that corresponded to the pressure in the alternate steady state. The second coefficient— b_2 —was relevant for the description of the vessel transition response between the alternate steady states. In our previous work [12], we demonstrated that these coefficients were highly sensitive parameters to the conditions of the blood vessel dynamics.

The mathematical analysis of the CPP dynamics showed no significant differences in the b_1 coefficients (Table 3) both in the control and in the test groups of animals. This result revealed that the maximal coronary perfusion pressure during the ischemic vasodilation was similar in both groups of animals. On the contrary, the b_2 coefficient in the test group was significantly higher compared to the b_2 coefficient of the control group, indicating the alteration in the dynamic response during the ischemic vasodilation in the presence of the glucagon. In addition, the time interval for the development of the maximal CPP during the alternate steady state was 125 seconds in the control group and it was significantly higher compared to the maximal CPP developmental period of 82 seconds in the test group. Therefore, these results demonstrated a faster increase of the coronary pressure in the glucagon-treated hearts (Table 3).

The values of $fAIV$ in the presence of the glucagon were significantly lower compared to the $fAIV$ values of the control group confirming the glucagon alteration of the ischemic vasodilation in our experimental design. On the other hand, the $fAIV$ values were not notably different from the $eAIV$ values in both groups, demonstrating the logical consequence of the extremely high coefficient of the correlation ($R^2 = 0.998 \pm 0.002$) between the fitted and the experimental CPP curves (Figure 2).

In addition, we calculated the Index of lipid peroxidation by measuring the levels of thiobarbituric acid reactive substances-TBARS (the known markers of the oxidative stress). Our data demonstrated significantly the elevated levels of TBARS within 2 minutes of the reperfusion both in the control and the test groups compared to the preischemic levels (Table 2). These findings were in accordance with the previous reports [1, 8, 32]. Also, our data demonstrated no apparent differences in the TBARS levels between the control and the test groups, during both the preischemic and the ischemia-reperfusion periods. These results indicated that the glucagon administration did not affect the TBARS levels in the isolated rat heart during both the preischemic and the ischemia-reperfusion periods in our experimental conditions.

Investigation of the NO level both before and during the ischemic vasodilation in the presence and in the absence of the glucagon clearly demonstrated that:

- (1) the NO levels during the preischemic period were significantly elevated in the presence of glucagon compared to the control group;
- (2) the NO level within the first 30 seconds of the reperfusion period was significantly higher, and after 90 seconds it was significantly lower in the test group, compared to the corresponding NO levels in the control group.

These findings were similar to the previous data reporting that the nitrite levels were higher under the glucagon treatment in the rat hepatocyte culture [33] and that the NO levels increased during the ischemia-reperfusion conditions, as well [7, 9, 34, 35]. Our experimental data also showed no apparent changes in the preischemic CPP values measured continuously during the glucagon administration. These results indicated that the elevated preischemic NO level in the test group did not significantly affect the coronary vessel diameters in our experimental conditions. Even though the elevated preischemic NO level did not have the significant effects on the histamine release, its role in influencing the mast cells stabilization [6] and consequently reducing the histamine release in the test group during ischemic vasodilation should not be neglected.

Our previous findings [12] demonstrated the influence of L-arginine on the faster development of the perfusion pressure in the isolated blood vessels. Although L-arginine in the previous investigation relaxed the smooth muscle in the blood vessel wall, the introduction of the hydrostatic pressure caused the blood vessel wall to become more rigid. These findings indicated that the increase of the NO release might not only induce the vasodilation, but it also might affect the biomechanical properties of the blood vessel wall. Even though the vasodilation occurred at the alternate steady states, the transient or the dynamic response of the blood vessel showed faster development of the pressure.

It is well known that the histamine release is elevated during the ischemic vasodilation and its concentration is increased during the ischemia [36]. Our results in this study confirmed these findings. Also, our results showed that the histamine liberation during the ischemic vasodilation was significantly reduced in the presence of glucagon in the isolated rat heart. These data were in the accordance with the previous reports describing the influence of the glucagon on the inhibition of the histamine release during the anaphylactic response in a guinea-pig isolated heart and the protective effects of the glucagon pretreatment during cardiac anaphylaxis [27].

Changes in both NO and histamine releases during both the ischemic vasodilation and the coronary autoregulation suggested a positive feedback between NO and histamine [11]. This may explain the biphasic NO release in our control group. The initial increase of NO within the first 30 seconds of the reperfusion period may lead to the increase of histamine release reaching the peak values between 30–90 seconds. Furthermore, the histamine stimulation of the endothelial histamine receptors produced the second peak of the NO release, by positive feedback mechanism, after 90 seconds of the reperfusion.

As mentioned earlier, the NO level during the preischemic period was significantly elevated in the presence of glucagon. At the same time, there was neither a change in the CPP nor in the basal (preischemic) histamine concentrations. Even though the preischemic NO level was significantly elevated, the amount of NO released seemed to be insufficient to dilate the coronary blood vessels, since there was no change in the CPP at that time. On the other hand, the increased amount of NO might be sufficient to stabilize mast

cells, therefore influencing the lack of the positive feedback relationship between NO and the histamine in the presence of glucagon. This could be the cause of the significantly lower NO release in the test group 90 seconds following the ischemic vasodilation.

5. Conclusions

In the summary, the influence of the glucagon on the dynamic responses of the coronary vessels of the isolated rat heart was uncertain during the ischemic vasodilation. In this study, our results showed a faster restoration of the coronary perfusion pressure during the reperfusion period in the presence of glucagon compared to the control group. Therefore, one of the possible postulated mechanism(s) explaining this process could be as following:

- (i) the glucagon increases NO release both during the preischemic period and within the first 30 seconds of the ischemic vasodilation;
- (ii) NO stabilizes the mast cells and prevents the histamine release during the ischemic vasodilation;
- (iii) further, the decreased histamine release then leads to the attenuated stimulation of the endothelial histamine receptor (H_1) and consequent decrease of the NO release, thus switching off this vasodilatory mechanism;
- (iv) simultaneously, the decreased histamine release leads to the attenuation of the H_2/H_3 mediated coronary vasodilation in the presence of glucagon;
- (v) as a result, the duration of the ischemic vasodilation is significantly shorter in the presence of glucagon;
- (vi) moreover, the increased NO release within the first 30 seconds of the ischemic vasodilation leads to the vasodilation in the presence of glucagon that in turn may also increase the rigidity of the coronary blood vessels, generating the faster development of CPP.

Finally, even though the mechanism(s) of the glucagon influences on the coronary vessels during the ischemic vasodilation is still unclear, our results clearly indicate the key involvement of both histamine and NO during this process.

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