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The effects of lactoferrin in a rat model of catecholamine cardiotoxicity

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Abstract Lactoferrin is recently under intense investigation because of its proposed several pharmacologically positive effects. Based on its iron-binding properties and its physiological presence in the human body, it may have a significant impact on pathological conditions associated with iron-catalysed reactive oxygen species (ROS). Its effect on a catecholamine model of myocardial injury, which shares several pathophysiological features with acute myocardial infarction (AMI) in humans, was examined. Male Wistar rats were randomly divided into four groups according to the received medication: control (saline), isoprenaline (ISO, 100 mg kg^{-1} s.c.), bovine lactoferrin (La, 50 mg kg^{-1} i.v.) or a combination of La + ISO in the above-mentioned doses. After 24 h, haemodynamic functional parameters were measured, a sample of blood was withdrawn and the heart was removed for analysis of various parameters. Lactoferrin premedication reduced some impairment caused by ISO (e.g. a stroke volume decrease, an increase in peripheral resistance and calcium overload). These positive effects were likely to have been mediated by the positive inotropic effect of lactoferrin and by inhibition of ROS formation due to chelation of free iron. The failure of lactoferrin to provide higher protection seems to be associated with the complexity of catecholamine cardiotoxicity and with its hydrophilic character.

Keywords Lactoferrin · Isoprenaline · Iron chelators · Reactive-oxygen species · Iron · Catecholamines

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Background

Lactoferrin is an innate iron-binding glycoprotein with many proposed, potentially positive pharmacological activities. It shares a high degree of homology with transferrin (Abdallah and El Hage Chahine 2000; Baker and Baker 2005; Metz-Boutigue et al. 1984). In the human body, transferrin acts as an

iron-transporting protein and is found predominantly in the blood, whereas lactoferrin is localized in exocrine secretes (e.g. saliva, tears, milk, bronchial mucus) and in the secondary granules of neutrophils (Weinberg 2003, 2006). Its role in man is only partially known. Lactoferrin inhibits the growth of many pathological microorganisms and its presence on mucosal surfaces represents the primary antimicrobial defence system of the organism. This antimicrobial property at least partially reflects its iron-binding capacity. Its affinity to iron is very high, about 260 times higher than that of transferrin (Baker et al. 1994) and, in contrast to transferrin, is able to retain iron under more acidic conditions (Abdallah and El Hage Chahine 2000).

The catecholamine model of myocardial injury possesses many pathophysiological similarities with acute myocardial infarction (AMI). The isoprenaline (ISO, synthetic catecholamine) model is therefore often used as a non-invasive model of AMI (Hasenfuss 1998; Chagoya de Sanchez et al. 1997; Rona 1985). The only possibility for myocardial tissue recovery in AMI represents the reperfusion of ischaemic myocardium. But the whole process, described as myocardial ischaemia-reperfusion (I-R), is associated with tissue derangement due to burst of hydroxyl radical catalyzed by free iron (Fenton reaction). In fact, studies confirmed increased levels of free intracellular iron and its release from ischaemic cells (Berenshtein et al. 2002; Coudray et al. 1994). Unfortunately, studies examining the effects of some iron chelators on I-R reported divergent results (Bolli et al. 1990; Reddy et al. 1991). Lactoferrin, which has been documented to inhibit the Fenton reaction (Gutteridge et al. 1981), may have some advantage in comparison to other iron chelators, in particular its endogenous origin.

Based on the aforementioned iron participation in the pathogenesis of myocardial damage, this work hypothesizes the potentially positive effects of lactoferrin in a catecholamine model of myocardial injury.

Methods

Animals

Young Wistar male rats obtained from Biotest s.r.o. (Konárovice, Czech Republic), weighing approximately 350 g, were used after 2 weeks of acclimatisation. The animals were maintained in an air-conditioned room and were allowed free access to a standard pellet diet for rodents and tap water. The study was performed under the supervision of the Ethical Committee of the Charles University in Prague, Faculty of Pharmacy in Hradec Králové. All experiments were performed in concordance with the guiding principles of laboratory animal care and use.

Study design

Animals were randomly divided into four groups:

control group (C, 7 animals)—received saline 1 ml kg^{-1} s.c.
isoprenaline group (I, 13 animals)—received 100 mg kg^{-1} of isoprenaline (ISO; Sigma–Aldrich,USA) in the aqueous solution s.c.
lactoferrin (La, 7 animals)—bovine lactoferrin 50 mg kg^{-1} (DMV International, USA) was administered i.v.
*lactoferrin + ISO (*LaI, 11 animals)—rats received lactoferrin i.v. 5 min before application of ISO in the same doses as above.

Saline and/or drug were administered 24 h before surgical procedure.

Experimental procedure

Animals were fasting for 12 h before the experiment and were anaesthetized with urethane (1.2 g. kg^{-1} i.p.; Sigma–Aldrich, USA). A PE catheter (0.5/1.0 mm filled with heparinized saline 50 IU ml^{-1}) was inserted into the right jugular vein for the injection of cold saline. A thermocatheter (o.d. 0.8 mm) was introduced through the left carotid artery into the aortic arc. Another PE catheter (0.5/1.0 mm filled with heparinized saline 50 IU ml^{-1}) was inserted into the left iliac artery, which was connected with the blood pressure transducer BPR-01 of the apparatus for measurement of haemodynamic variables Cardiosys® (Experimentria Ltd, Hungary) with software Cardiosys V 1.1. This device uses the thermodilution transpulmonary method according to the Stewart–Hamilton principle (Spiller and Webb-Peploe 1985).

The first measurement was carried out following 15 min equilibration period after the surgical

procedure. Functional variables were obtained in a total of four times in 5 min intervals. Results are expressed as an index (variable divided by the body weight) except for blood pressure, heart rate and "double product" (mean blood pressure multiplied by heart rate). The last parameter is commonly used as an index of cardiac oxygen consumption (Lentner 1990). Peripheral resistance was calculated as the mean arterial blood pressure divided by cardiac output, and cardiac power as a product of the mean blood pressure and cardiac output.

Following haemodynamic measurements, approximately 5 ml of blood was withdrawn from the abdominal aorta to the heparinized test tube (170 IU). The animal was then sacrificed by i.v. KCl overdose (1 mM), after which the heart ventricles were excised, weighed and frozen at −20°C for further analysis of ion content.

Histological analysis

After the autopsy, a routine histological examination of cardiac ventricular tissue was performed. Tissue blocks of the transversely sectioned left and right ventricles (the region under the atria with heart apex) were fixed by immersion in 4% paraformaldehyde for 3 days. Furthermore, ten consecutive longitudinal paraffin sections through the ventricles were cut (7 μm in thickness) and subsequently stained with haematoxylin-eosin.

Biochemical parameters

Cardiac troponin T (cTnT) and vitamin E were measured in serum, malondialdehyde (MDA) was measured in plasma, antioxidant enzymes were measured in erythrocytes and total glutathione (GSH) was measured in the whole blood. cTnT was determined by electrochemiluniscence immunoassay (Elecsys 2010, Roche Diagnostics), which employs two monoclonal antibodies specifically directed against cTnT. MDA was measured as a red complex with thiobarbituric acid at 485, 532 and 560 nm using Beckman DU 640 spectrophotometer (Beckman, Palo Alto, USA). Capillary electrophoresis was used for the separation of glutathione, which was then measured by UV detection (System P/ACE 5100, Beckman) at 200 nm. Glutathione peroxidase (GPx) was determined spectrophotometrically using a commercial kit (Ransel, Randox, UK) according to the manufacturer's instruction as a decrease of absorbance at wavelength 340 nm (Cobas Mira, Roche, Switzerland). Superoxide dismutase (SOD) was determined spectrophotometrically at 505 nm using a commercial kit (Ransod, Randox, UK). After deproteinization, the analysis of vitamin E (α-tocopherol) with fluorimetric detection was performed in a HPLC system HP1050 (Hewlett Packard, Germany).

Microelements in the heart tissue

Frozen samples of myocardial tissue were dried, weighed and digested by microwave digestion using nitric acid and hydrogen peroxide (Milestone MLS 1200 MEGA, Italy). Iron, copper and selenium were determined using graphite furnace atomic absorption spectrometry (Unicam, Solaar 959, UK), zinc was determined using flame atomic absorption spectrometry (Unicam, Solaar 959, UK) and calcium was measured photometrically using flame photometry (Eppendorf, Efox 5053, Germany). Results are expressed as μmol g^{-1} (iron, copper, zinc, calcium) or nmol g^{-1} (selenium) of dry tissue.

Statistics

Data are expressed as means ± SEM. Groups were compared by one-way ANOVA followed by Tukey's Multiple Comparison Test by means of GraphPad Prism version 4.00 for Windows, GraphPad Software (San Diego, California, USA). Differences between groups were considered to be significant when $P \leq 0.05$, unless indicated otherwise.

Results

Final numbers of rats were seven in control, lactoferrin (La) and lactoferrin + ISO (LaI) groups and nine in the ISO group (because of high results variability). None of the control animals or animals from the lactoferrin group died. One rat (group LaI) died during surgery. There was comparable mortality in I group and combined group LaI (four and three animals, respectively). Functional parameters are summarized in Fig. 1 and Table 1.

Control and lactoferrin treated animals had negligible levels of serum cTnT and there was no

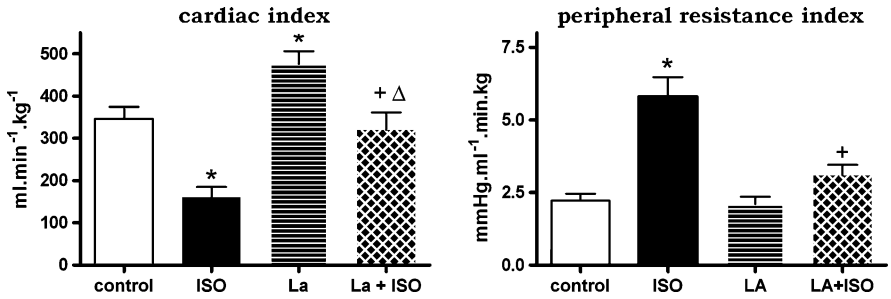

Fig. 1 Stroke volume index and peripheral resistance index 24 h after drug application. $^{*}P < 0.05$ versus control, $^{+}P < 0.05$ versus ISO, $^{\Delta}P < 0.05$ versus La

Table 1 Functional parameters measured 24 h after drug(s) administration

Parameter/group	Control	Isoprenaline (ISO)	Lactoferrin (La)	La + ISO
Heart rate (beats min^{-1})	418 ± 10	453 ± 7*	429 ± 9	470 ± 10*,***
Diastolic blood pressure (mmHg)	110 ± 6	121 ± 9	135 ± 7	127 ± 6
Systolic blood pressure (mmHg)	92 ± 4	102 ± 7	112 ± 5	107 ± 6
Mean blood pressure (mmHg)	82 ± 4	93 ± 7	101 ± 5	98 ± 6
Stroke volume index (ml kg^{-1})	0.83 ± 0.07	0.36 ± 0.06*	1.19 ± 0.12*	0.69 ± 0.09**,***
Double product (mmHg beats min^{-1})	38,215 ± 1,644	46,070 ± 3,652	46,250 ± 3,523	50,319 ± 2,443*
Cardiac power index (mmHg ml kg^{-1} min^{-1})	31,511 ± 2,947	15,917 ± 2,239*	52,504 ± 3,490*	34,481 ± 4,987**,***

Results are expressed as mean ± SEM. * $P < 0.05$ versus control, ** $P < 0.05$ versus ISO, *** $P < 0.05$ versus La

statistical difference between myocardial calcium levels in these groups. Isoprenaline brought about a marked cTnT release and myocardial calcium overload. Lactoferrin premedication did not affect the release of cTnT; however, it decreased calcium overload caused by ISO (Fig. 2). Heart ventricle weight index was elevated in the ISO group when compared to the control or La groups, and contrarily, lactoferrin premedication rather worsened this increase (Fig. 3).

There was no statistical significance in myocardial iron level (Table 2). Similarly, no statistical significance was found in myocardial copper, selenium or zinc concentrations (Table 2).

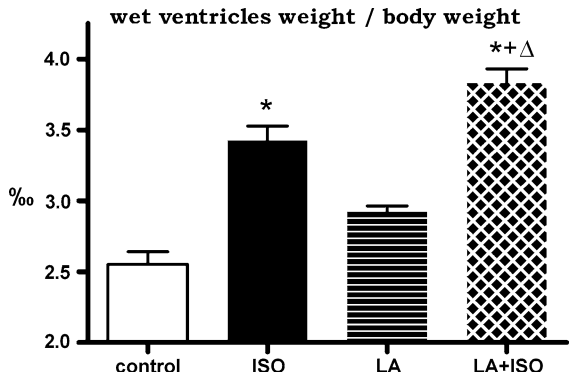

Fig. 3 Wet ventricles weight index $^{*}P < 0.001$ vs. control, $^{+}P < 0.05$ versus ISO, $^{\Delta}P < 0.001$ versus La

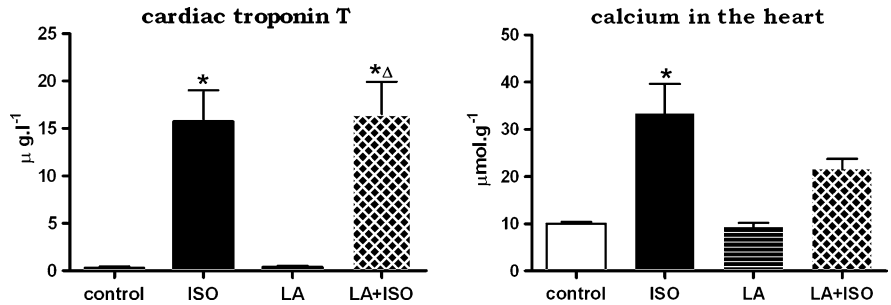

Fig. 2 Cardiac troponin T in serum and calcium concentration in the myocardial tissue. $^{*}P < 0.01$ versus control, $^{\Delta}P < 0.01$ versus La

Table 2 Biochemical parameters and myocardial element content 24 h after drug administration

Parameter/group	Control	Isoprenaline (ISO)	Lactoferrin (La)	La + ISO
TBARS (μmol l^{-1})	1.3 ± 0.1	1.6 ± 0.2	1.0 ± 0.1	1.4 ± 0.1
SOD (U g^{-1} of Hb)	3,092 ± 253	3,288 ± 387	2,461 ± 266	2,138 ± 150*·**
Vitamin E (μmol l^{-1})	9.0 ± 0.4	8.0 ± 0.5	8.2 ± 0.7	7.9 ± 0.7
Copper (μmol g^{-1} of tissue)	0.58 ± 0.05	0.72 ± 0.03	0.76 ± 0.09	0.83 ± 0.07
Iron (μmol g^{-1} of tissue)	3.8 ± 0.2	3.6 ± 0.2	4.2 ± 0.1	4.2 ± 0.2
Zinc (μmol g^{-1} of tissue)	1.09 ± 0.01	1.09 ± 0.01	0.93 ± 0.08	1.13 ± 0.07
Selenium (nmol g^{-1} of tissue)	7.8 ± 1.2	8.6 ± 1.0	11.3 ± 1.5	11.9 ± 1.5

TBARS thiobarbituric acid reactive substances in plasma, *SOD* superoxide dismutase in erythrocytes; vitamin E in serum; copper, iron, selenium and zinc in the myocardial tissue

Results are expressed as mean ± SEM. * $P < 0.05$ versus control, ** $P < 0.05$ versus ISO

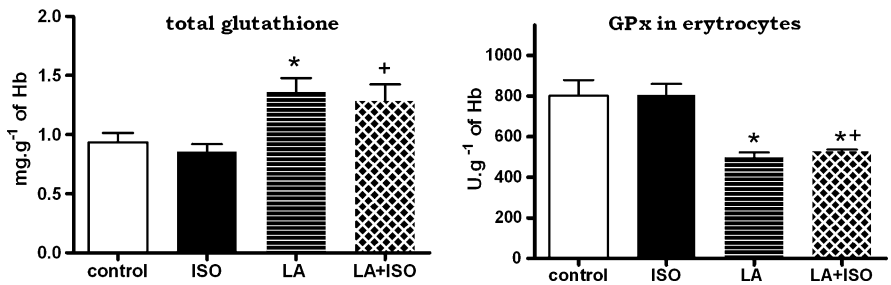

Fig. 4 Total glutathione and glutathione peroxidase (GPx) in erythrocytes. *$P < 0.05$ versus control, $^{+}P < 0.05$ versus ISO

Lactoferrin significantly elevated levels of total glutathione while significantly decreasing erythrocyte GPx and insignificantly SOD in healthy animals (Fig. 4; Table 2). Moreover, lactoferrin tended to decrease plasma thiobarbituric acid reactive substances (TBARS) but this decrease was not statistically significant (Table 2). Other results concerning markers of ROS and antioxidants are shown in Table 2.

The normal structure of myocardium was found in intact control animals (Fig. 5a). Comparable findings were also visible after administration of lactoferrin (Fig. 5b). In the ISO group (Fig. 5c), severe acute diffuse toxic damage with an inflammatory reaction was found in the whole myocardium (with a maximum in the subendocardial region, particularly in the heart apex). Myocytes with intensely eosinophilic cytoplasm prevailed, and scattered pyknotic nuclei were also seen. Mild inflammatory infiltrate and macrophages removing the debris of necrotic cells, as well as slight interstitial oedema, were also observed. The administration of lactoferrin (Fig. 5d) did not affect morphological changes in myocardium induced by ISO.

Discussion

The administration of ISO, a synthetic catecholamine with non-selective beta-agonist activity, leads to tachycardia and hypotension, which in sufficient doses, is associated with ischaemia followed by marked damage of the myocardium. This resembles, in some aspects, the AMI in man (Chagoya de Sanchez et al. 1997; Rona 1985). Ischaemia alters iron homeostasis and redox-active free (unbound or loosely bound) iron, which catalyses ROS-generation upon oxygen delivery restoration, appears in the circulation as well as intracellularly (Berenshtein et al. 2002; Coudray et al. 1994). An increase in the concentration of free iron in the myocardium may accompany certain medical procedures, and can be prevented by administration of apo-transferrin, a protein with similar structure to lactoferrin (Parkkinen et al. 2006). Moreover, lactoferrin has a much higher affinity to iron compared with transferrin and was shown to inhibit iron-catalysed ROS formation and decrease ischaemia-reperfusion injury of corneal epithelial cells (Britigan et al. 1994; Shimmura et al. 1998). Some synthetic iron chelators

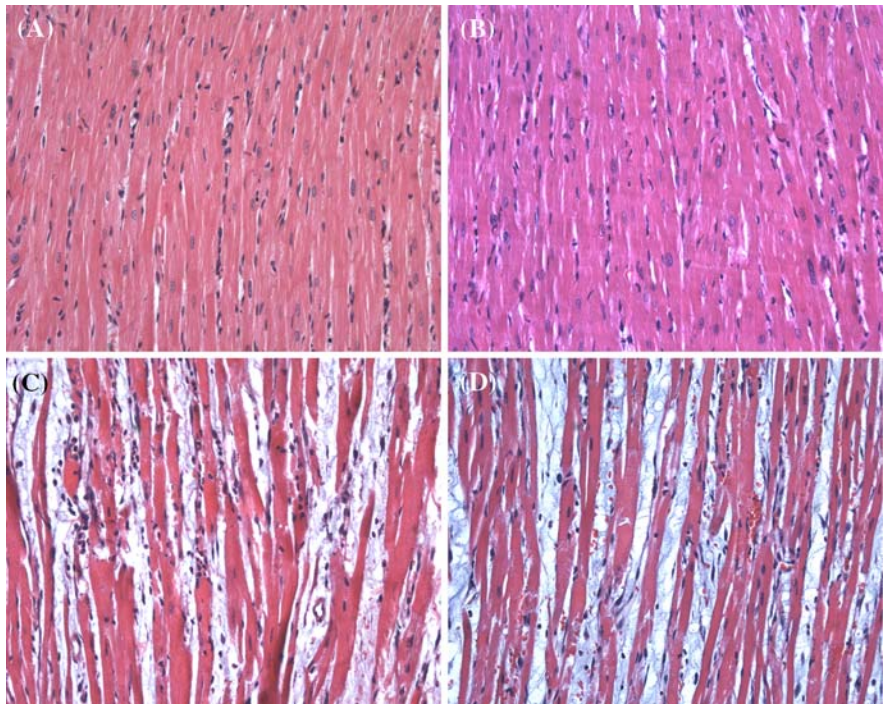

Fig. 5 Histological examination of myocardium of all groups. In both control (**a**) and lactoferrin treated rats (**b**) unaffected myocardium was found: cardiomyocytes with centrally located oval-shaped nuclei and cytoplasm filled with cross-striated myofibrils. On the other hand, severe damage of cardiomyocytes resulted in their degeneration/necrosis, seen in both isoprenaline and isoprenaline + lactoferrin treated groups (**c**, **d**). Moreover inflammatory infiltrate and as interstitial oedema were present in this initial stage of myocardial damage. Lactoferrin treatment did not affect morphological changes caused by the administration of isoprenaline. Haematoxylin–eosin. Direct magnification: 200×

have been well documented to alleviate the toxic effects of excess iron in various pathological conditions, where abundant iron may participate on ROS propagation (Kalinowski and Richardson 2007; Kontoghiorghes 2006; Sterba et al. 2007). Similarly, this study documented partially protective effects of lactoferrin on catecholamine-mediated injury.

In contrast to transferrin, serum lactoferrin concentration is generally low. It rises significantly after exercise, sometimes reaching more than 5 μg ml^{-1}, and in burnt patients may even reach up to 40 μg ml^{-1} (Fielding et al. 2000; Inoue et al. 2004; Wolach et al. 1984). In our study the serum lactoferrin levels were not measured; however, due to intravenous administration it can be assumed that its maximum concentration may reach about 1,500 μg ml^{-1}, which significantly exceeds physiological levels of endogenous lactoferrin. On the other hand, it is only about one half of normal human serum transferrin concentration (3,150 μg ml^{-1}) (Plomteux et al. 1987). Lactoferrin has a very short initial plasma half-life of about 2 min and, therefore, only 15% of the administered dose remained in the plasma after 10 min. This first rapid disappearance corresponds to its distribution in the liver and partly in the spleen. Only about 1% of the administered dose remained in plasma after 10 h (Bennett and Kokocinski 1979; Karle et al. 1979; Regoeczi et al. 1985; Ward et al. 1983). Based on these data, it may be concluded that high initial plasma levels of lactoferrin promptly dropped and were in physiologically relevant concentration ranges during the experiment.

There is some controversy concerning the role of lactoferrin in inflammation. On one hand, elevated levels of lactoferrin are found in patients with ischaemic stroke, and lactoferrin is known to promote leukocyte adhesion to the endothelial cells and to cause extravasation of plasma (Erga et al. 2000; Kurose et al. 1994; Oseas et al. 1981; Santos-Silva et al. 2002). On the other hand, lactoferrin release induced by leukocyte activation does not seem to amplify inflammation but, contrarily, to reduce the

consequences of ROS generation during inflammation (Ward et al. 1983; Weinberg 2003). Especially, apo-lactoferrin released from neutrophils (iron-free lactoferrin) appears to have the most potent inhibitory effect on ROS-formation (Raghuveer et al. 2002; Weinberg 2003).

The dose used in this study (50 mg kg^{-1} i.v.) was not apparently pro-inflammatory in contrast to our preliminary experiments with higher doses ($\geq$100 mg kg^{-1} i.v.). Moreover, lactoferrin had a tendency to decrease plasma TBARS and to increase total blood glutathione in comparison with healthy animals. There was an increase in wet ventricle weight in the lactoferrin group. However, such an increase was neither associated with obviously conspicuous morphological changes nor with an increase in serum cTnT concentration, thus suggesting its harmlessness. The only suspicious features are a significant decrease in erythrocyte GPx and a non-significant decrease in erytrocyte SOD. Erythrocyte enzymes are generally less susceptible to acute changes and indeed, ISO administration alone did not cause any change. Lactoferrin binds to various surface molecules and its interaction with some type of blood cells has been well documented (Britigan et al. 1994; Brock 2002). Therefore, it seems to be possible that these measurements may be blunted by lactoferrin binding to erythrocytes. This assumption must be verified with further examination. Generally, it may be inferred that pro-inflammatory effects of bovine lactoferrin in this study were negligible and of transient character.

Other observed effects of lactoferrin on healthy animals are not linked to its pro/anti-inflammatory properties, such as increased stroke volume and related cardiac indices. No change in the peripheral resistance and rather an increase in diastolic blood pressure, indicated that lactoferrin increased myocardial contractility (Fig. 1; Table 1). This positive inotropic effect is very likely to be responsible for the partial amelioration of cardiovascular impairment caused by ISO. ROS has been well documented to impair physiological vascular function (Paffett and Walker 2007). Lactoferrin binds extracellular free iron and subsequently inhibits ROS formation. Therefore, the inhibition of increasing peripheral resistance, together with a tendency to decrease myocardial calcium overload, is very likely to be related to the iron chelation properties of lactoferrin. Insignificant differences in myocardial iron concentrations are not very surprising. Levels of released iron both during and after ischemic insult seem to be very small, albeit sufficient for ROS production. Therefore iron chelation in such conditions influences intracellular levels only marginally, in contrast to iron overloaded cells. Similarly, iron-loaded lactoferrin produces an increase in intracellular iron levels (Shimmura et al. 1998; van Snick et al. 1977).

Pathogenesis of catecholamine cardiotoxicity is multifactorial and not fully understood. It involves ROS-generation and adrenergic stimulation (Neri et al. 2007; Rona 1985). The first mechanism can be affected by iron chelation but the latter only partially, if at all. This may, at least in part, explain the failure of lactoferrin to decrease other catecholamine cardiovascular damage. The other important factor is the hydrophilic nature of lactoferrin and thus its limited intracellular penetration.

Conclusion

Our experiment did not show that the prophylactic administration of bovine lactoferrin (50 mg kg^{-1} i.v.), may reverse rat myocardial injury caused by s.c. administration of necrogenic dose of ISO within a 24-h period. Its partial protective effects are likely based on: (1) an unknown positive inotropic mechanism, which increases stroke volume index in healthy animals and therefore inhibits the drop in this parameter in catecholamine-treated animals; (2) extracellular iron chelation that inhibits an increase in peripheral resistance caused by ISO insult. The failure of lactoferrin to provide a greater protection against catecholamine cardiotoxic injury is likely to be associated mainly with its hydrophilic character and with the complexity of catecholamine cardiotoxicity. Further examination following this pilot study, which for the first time evaluated the direct effects of lactoferrin on cardiovascular function in healthy and ISO-treated animals, is encouraged. One possible method may be the conjugation of lactoferrin with non-toxic polymers (Ward et al. 1983), which may enable a smaller initial dose of lactoferrin and the prolongation of lactoferrin elimination half-time.

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