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Plasma Fibronectin During Myocardial Ischemia-Reperfusion: Effects of Magnesium, Diltiazem, and a Novel Mac-1 Inhibitor

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The important role of fibronectin (Fn) has been recognized in patients with ischemic heart disease. However, serial changes of Fn during both brief and prolonged ischemia-reperfusion are poorly known. Plasma Fn was measured during acute myocardial infarction (AMI) and myocardial stunning (MS), and in the absence of myocardial injury. The effects of magnesium (Mg), diltiazem, and a Mac-1 inhibitor on the level of Fn were elucidated. Forty-nine swine underwent prolonged (50 min) or brief (8 min) coronary artery occlusion followed by reperfusion, while six control animals were free of ischemia. During the AMI experiments, plasma Fn underwent a significant progressive increase. Mg or diltiazem similarly affects the plasma Fn, reducing its release during the entire reperfusion period, and did not influence the plasma Fn in the absence of myocardial injury. Contrarily, Mac-1 inhibition resulted in the Fn elevation in controls, and during the occlusion phase, with no significant effect during reperfusion. There were no changes in the plasma Fn during MS, while inhibition of Mac-1 was associated with the significant increase of Fn during ischemia-reperfusion. Ability of Mg, diltiazem, and leumedins to modulate plasma Fn level may have direct clinical implications for the use of these agents in patients with coronary artery disease. Am. J. Hematol. 57:309–314, 1998.

Key words: fibronectin; acute myocardial infarction; myocardial stunning; magnesium; diltiazem; Mac-1 inhibitor; animal model

INTRODUCTION

Fibronectin (Fn) is a high molecular-weight dimeric glycoprotein, which exists in tissue and plasma forms [1]. Although evidence concerning the role of Fn in hemostasis is far from conclusive, it appears that this protein enhances platelet adhesion and spreading on exposed vascular matrix components [2]. The source of this elevated Fn during myocardial injury is unknown; however, there are several plausible mechanisms. Both platelets [3] and neutrophils [4], upon activation, have been shown to release significant amounts of fibronectin. Another potential source of Fn is located in the subendothelial compartment, which becomes vulnerable to degradation after endothelial damage.

Since vasoreactivity is an important factor in the modulation of myocardial ischemia, the present study was designed to explore serial changes of the plasma Fn during acute myocardial infarction (AMI) and myocardial stunning (MS).

There has been some debate regarding the benefit of magnesium (Mg) and diltiazem in the treatment of AMI, due to conflicting results from clinical trials [5–8]. Several different hypotheses have been advanced to explain the cardioprotective properties of Mg, including some evidence that Mg could act as a ''natural'' Ca-channel

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blocker, preventing calcium flux [9]. Analysis of the patterns of Mg and diltiazem on the plasma Fn during AMI are exploratory.

The leumedins are a class of small organic molecules with activity in a number of animal models of inflammation. N-[9H-(2,7-dimethylfluorenyl-9-methoxy)-carbonyl]-L-leucine (NPC 15669) is an inhibitor of leukocyte recruitment [10]. NPC 15669 inhibits Mac-1 binding to the endothelium [11]. Mac-1 (CD11b/CD18), which is found on neutrophils, is involved in the ischemia-reperfusion phenomenon [12]. Favorable effects of this novel leumedin have also been shown following both brief and prolonged episodes of ischemia-reperfusion in a porcine model of MS and AMI [13]. The immediate effect of leumedins on the plasma Fn in the setting of myocardial ischemia-reperfusion is currently unknown.

MS is a transient state of contractile dysfunction occurring subsequent to an episode of ischemia followed by reperfusion [14,15]. MS is generally considered as a form of reperfusion injury, related to re-opening of briefly occluded coronary artery, which is common in patients with coronary artery disease [16,17]. Clinically, this phenomenon may contribute to left ventricular pump failure and, therefore, may carry risks of both morbidity and mortality [18]. One of the theories explains MS phenomenon as a burst of free radicals formed within the first minute of reperfusion [19]. The alternate hypothesis is that cytosolic calcium overload damages mechanisms for normal intracellular calcium regulation [14,15]. A further component of reperfusion injury, under active investigation, is endothelial damage with alterations of platelet function and vasoreactivity [20]. While it has been established that both coronary and systemic plasma concentrations of Fn increase following reperfusion injury, there is lack of evidence regarding their plasma levels after Mg, diltiazem, or NPC-15669 therapy.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee of the University of Maryland. All procedures conformed to the guidelines established by the U.S. Department of Health and Human Services, published by the U.S. National Institute of Health (NIH publication no. 85-23, revised 1985).

Animals

Seventy-five purebred Yorkshire female swine (34–41 kg weight) 10–12 weeks of age were housed at our institution for a minimum of 1 week prior to use. They received normal swine food (Purina, Richmond, IN). The animals were randomized into seven groups. The first four groups underwent prolonged coronary artery occlusion, which resulted in the development of AMI. The first group received intracoronary 250 mg $MgSO_4$ (25% of the usual intravenous dose) (Gensia Laboratories, Irvine, CA) with the onset of reperfusion and lasting 12 min. The second group received intracoronary 2.5 mg of diltiazem at the rate of 5.6 $\mu g/kg/min^{-1}$ (25% of the established intravenous dose) (Cardizem®; Marion Merrell Dow Inc., Kansas City, MO) beginning with the onset of reperfusion period and lasting 12 min. The third group were pretreated with the intravenous bolus of 10 mg/kg of NPC 15669 followed by a maintenance dose of 6 $mg/kg/h^{-1}$ during the entire ischemia-reperfusion period. The fourth group received normal saline. The next two groups underwent brief coronary artery ischemia, which resulted in the development of mild MS. The fifth group received a bolus of 10 mg/kg of NPC 15669 (Scios Nova, Inc., Mountain View, CA) at the beginning of occlusion, followed by a maintenance dose of 6 $mg/kg/h^{-1}$ during of reperfusion phase. The same dose has been used in the other experimental studies with this compound (17, 20, 42, 44). NPC was diluted in deionized water, and administered into the femoral vein using an infusion pump (Harvard Apparatus, South Natick, MA). The sixth group received intravenous saline and served as the reference for the reperfusion injury experiments. The seventh group received the same doses of diltiazem, Mg, and NPC 15669 as in the AMI groups, but animals were free of coronary artery ligation. Plasma Fn was measured at the same prespecified time points as in the AMI swine population.

Myocardial Infarction and Stunning Model

A detailed protocol for the AMI [20,21] and MS [20,22] experiments can be found elsewhere. Briefly, for AMI experiments, all animals were subjected to 50 min of the LAD occlusion, followed by 3 hr of reperfusion. Hemodynamics (mean arterial pressure, heart rate, and left ventricular end-diastolic pressure) were continuously monitored through the entire protocol. Infarct size was documented by vital staining of myocardium with Tetrazolium red. For MS studies, left anterior descending (LAD) coronary artery was occluded for 8 min followed by 90 min of reperfusion. MS phenomena has been evaluated by the significant changes of myocardial contractility, which was assessed by epicardial Doppler displacement probes. Stunning time was defined as the duration from the start of reperfusion, comprising depressed contractility, to the point of recovery.

Sample Collection and Analysis

Blood was collected six times from the femoral vein during the AMI protocol: at the baseline; at 25 and 50 min of occlusion; and after 60, 120, and 180 min of reperfusion. Blood was collected five times during the MS experiments: at the baseline; at 4 and 8 min of occlusion; and at 60 and 90 min of reperfusion. To avoid possible observer bias, blood samples were coded and

TABLE I. Characteristics of the Animal Groups That Underwent Myocardial Ischemia-Reperfusion Experiments and Controls†

A. Acute myocardial infarction

Group	Weight (kg)	Enrolled	Excluded	Analyzed	Infarct size (g/kg body wt)
Magnesium	35.9 ± 0.6	11	4	7	0.16 ± 0.05*
Diltiazem	37.8 ± 0.7	11	3	8	0.13 ± 0.06*
NPC 15669	36.1 ± 0.8	13	5	8	0.21 ± 0.07*
Saline	37.5 ± 0.9	14	4	10	0.42 ± 0.04
Controls	36.4 ± 0.7	6	0	6	n/a

B. Myocardial stunning

Group	Weight (kg)	Enrolled	Excluded	Analyzed	Stunning time (min)
NPC 15669	34.8 ± 1.0	10	2	8	26.7 ± 4.0*
Saline	36.6 ± 0.7	10	2	8	50.0 ± 4.3

†n/a, nonapplicable.
*$P < 0.05$ when compared with controls.

blinded before any measurements. Plasma Fn was determined by an individual unaware of the experimental protocol.

Fibronectin was measured in EDTA-treated platelet-poor plasma (500*g* for 5 min) by kinetic turbidimetry of the antigen-antibody-reaction according to the principle of the fixed time method (Boehringer Mannheim, Mannheim, Germany).

All comparisons were done using repeated measures ANOVA. A post hoc comparison using the Bonferroni *t*-test was performed to identify specific differences between the baseline Fn values and those of ischemia-reperfusion. The values are expressed as mean ± SEM; $P < 0.05$ was considered significant.

RESULTS

Exclusion of Animals

Characteristics of the experimental groups are presented in Table I.

Controls. All six pigs survived intravenous drug infusions.

Acute myocardial infarction. Of the 49 swine, 12 animals were excluded for ventricular fibrillation, which occurred during the end of occlusion or at early reperfusion. Four pigs did not sustain an infarction and were considered atypical of the overall study design. After these exclusions, the remaining 33 animals were analyzed for the serial changes of the plasma Fn.

Myocardial stunning. All 20 pigs under investigation survived the MS experiment. None of them developed ventricular fibrillation. However, 4 animals were excluded for the Doppler probe instrumentation failure.

Hemodynamic Variables

Table II summarizes the hemodynamic parameters. There were no changes in hemodynamics after intravenous infusion of diltiazem, Mg, and NPC 15669 in the saline-treated animals. There were no significant difference in heart rate, left ventricular end-diastolic pressure (LVEDP), or left ventricular systolic pressure between saline and drug-treated groups at baseline, during occlusion, or during the first 120 min of reperfusion. After 180 min of reperfusion, the Mg group had a lower LVEDP and tended to have a higher heart rate than animals from the other groups.

Plasma Fibronectin

At baseline, there were no differences between the experimental groups. However, intravenous drug infusions, as brief and prolonged myocardial ischemia, followed by reperfusion, were associated with the significant disturbances in the plasma Fn measured in the systemic circulation. The cumulative data on the plasma Fn during AMI and MS are summarized in Table III.

Controls

There were no changes in the plasma Fn levels after administration of Mg or diltiazem. However, NPC 15669 infusion was associated with the immediate twofold increase of the plasma Fn, which remained significant for 2 hr after Mac-1 blockade.

Acute Myocardial Infarction

Saline. Plasma Fn increased significantly during coronary artery occlusion and remained elevated during reperfusion when compared to the baseline.

Mg group. Plasma Fn underwent a significant increase during occlusion. However, a marked decrease in the plasma Fn concentration has been observed during the entire reperfusion phase.

Diltiazem group. The pattern of the serial changes of plasma Fn in the diltiazem group were very similar to those that occurred in Mg-treated swine. At any time point during ischemia-reperfusion, there were no differ-

TABLE II. Hemodynamics During Experiments on Myocardial Infarct Size*

	Mg			Diltiazem		
Time point	MAP (mmHg)	HR (beats/min)	LVEDP (mmHg)	MAP (mmHg)	HR (beats/min)	LVEDP (mmHg)
Baseline	49.98 ± 2.96	99.00 ± 9.54	6.8 ± 1.63	43.32 ± 3.05	102.00 ± 6.80	6.33 ± 1.58
25′ Occl	47.13 ± 3.35	85.00 ± 6.12	10.80 ± 2.42	42.35 ± 1.32	85.00 ± 7.64	10.67 ± 1.33
50′ Occl	41.80 ± 0.73	87.00 ± 4.36	11.60 ± 1.60	39.10 ± 2.55	90.00 ± 3.65	8.33 ± 2.15
10′ Rep	42.97 ± 2.18	89.00 ± 5.10	10.80 ± 2.87	38.77 ± 2.01	87.00 ± 11.67	8.00 ± 3.85
15′ Rep	43.67 ± 1.04	89.00 ± 5.10	12.40 ± 1.47	40.10 ± 1.74	88.00 ± 4.01	12.67 ± 4.83
60′ Rep	41.10 ± 1.73	81.00 ± 6.20	13.20 ± 2.15	38.45 ± 1.32	78.00 ± 2.79	6.67 ± 1.91
120′ Rep	38.10 ± 0.49	89.00 ± 9.66	13.75 ± 1.75	38.00 ± 1.01	77.00 ± 3.74	9.60 ± 5.18
180′ Rep	36.70 ± 2.26	105.00 ± 25.00	4.00 ± 2.00	36.00 ± 2.04	70.00 ± 5.24	9.60 ± 2.04
	NPC-15669			Saline		
Time point	MAP (mmHg)	HR (beats/min)	LVEDP (mmHg)	MAP (mmHg)	HR (beats/min)	LVEDP (mmHg)
Baseline	46.11 ± 3.27	90.00 ± 8.14	6.46 ± 2.78	49.58 ± 4.05	93.00 ± 9.95	6.80 ± 2.33
25′ Occl	43.07 ± 4.57	84.00 ± 7.23	8.90 ± 1.66	44.88 ± 4.43	88.00 ± 8.93	9.60 ± 1.83
50′ Occl	40.62 ± 3.69	83.00 ± 6.26	9.03 ± 2.28	43.88 ± 4.87	85.00 ± 8.19	8.60 ± 1.78
10′ Rep	42.29 ± 3.44	80.00 ± 6.92	11.40 ± 2.03	44.16 ± 4.33	83.00 ± 7.68	12.80 ± 1.20
15′ Rep	43.61 ± 4.17	86.00 ± 7.32	12.07 ± 2.32	44.12 ± 3.34	88.00 ± 10.20	12.40 ± 1.94
60′ Rep	40.29 ± 3.31	79.00 ± 7.02	13.40 ± 1.21	45.08 ± 4.69	84.00 ± 8.72	13.00 ± 0.89
120′ Rep	39.94 ± 3.57	78.00 ± 5.36	14.21 ± 1.72	41.40 ± 3.82	79.00 ± 6.52	14.80 ± 2.24
180′ Rep	38.11 ± 2.80	77.00 ± 6.49	12.10 ± 3.08	41.46 ± 3.34	74.60 ± 5.31	14.60 ± 2.68

*Values are mean ± SEM. Occl, occlusion; Rep, reperfusion; MAP, mean arterial pressure; HR, heart rate; LVEDP, left ventricular end-diastolic pressure.

TABLE III. Serial Changes of the Plasma Fibronectin Level During Myocardial Ischemia-Reperfusion and Control Animals†

A. Control (no myocardial injury)

Drug	Baseline	25′	50′	110′	170′	230′
Mg	53.64 ± 2.33	58.24 ± 4.11	56.60 ± 4.29	60.09 ± 3.61	53.27 ± 4.50	50.89 ± 4.46
Diltazem	54.11 ± 2.90	51.83 ± 4.55	55.31 ± 4.80	57.33 ± 3.48	55.20 ± 3.77	52.65 ± 3.41
NPC	54.06 ± 3.26	121.70 ± 5.86*	103.32 ± 5.42*	72.19 ± 3.64*	55.21 ± 3.70	57.11 ± 2.96

B. Acute myocardial infarction

Group	Baseline	25′ occl.	50′ occl.	60′ reperf.	120′ reperf.	180′ reperf.
Saline	54.19 ± 2.66	93.56 ± 4.14*	139.17 ± 3.48*	146.60 ± 1.77*	145.52 ± 2.09*	146.50 ± 1.85*
Mg	53.17 ± 1.08	94.86 ± 4.66*	141.07 ± 4.76*	111.33 ± 2.81*,**	114.02 ± 4.56*,**	109.50 ± 4.54*,**
Diltazem	52.83 ± 2.15	96.03 ± 4.04*	142.19 ± 4.88*	117.06 ± 2.58*,**	106.66 ± 3.86*,**	118.51 ± 3.03*,**
NPC	63.07 ± 4.42	171.82 ± 4.92**	140.67 ± 2.77*	138.67 ± 3.57	137.67 ± 2.14	137.67 ± 1.26

C. Myocardial stunning

Group	Baseline	4′ occl.	8′ occl.	60′ reperf.	90′ reperf.
Saline	53.17 ± 1.08	53.00 ± 0.58	52.33 ± 1.71	51.33 ± 1.49	52.54 ± 1.41
NPC	54.74 ± 3.81	77.38 ± 2.29*,**	91.06 ± 1.98*,**	88.47 ± 2.44*,**	93.30 ± 1.77*,**

†Data expressed as mean ± SEM (μg/ml). occl., occulsion; reperf., reperfusion.
*Significant difference when compared to baseline.
**Significant difference between identical time points during ischemia-reperfusion as compared to the saline-treated animals.

ences in the plasma Fn when compared with the Mg group.

NPC 15669 group. Similar to the saline group, the Fn level underwent a significant increase during occlusion, which was recorded throughout entire reperfusion. However, there was a major difference in terms of a marked increase in the plasma Fn concentration in the NPC 15669 group at the beginning of occlusion, when compared with the saline-treated swine.

Myocardial Stunning

Saline. There were no differences in the plasma Fn profile during ischemia-reperfusion when compared to the baseline.

NPC 15669 group. The patterns of the plasma Fn reveal rapid, progressive, and an almost twofold elevation of the plasma Fn beginning at the early occlusion phase and lasting the entire reperfusion.

DISCUSSION

The current study suggests that prolonged coronary artery occlusion followed by reperfusion in the open-chested swine model is associated with significant changes in the plasma Fn. The exact mechanism of such action remains unknown.

Despite general agreement on the importance of Fn in coronary artery disease and myocardial ischemia, the significance of plasma fibronectin levels is a matter of considerable controversy. Our interest in the determination of plasma Fn levels during MS was stimulated by recent reports of associated leumedins and Fn during ischemia-reperfusion. Plasma Fn levels were shown to be significantly decreased during the reperfusion in patients with AMI complicated by ventricular arrhythmia, left ventricular failure, or death. However, plasma Fn level remains unchanged in patients following uncomplicated AMI [23]. Meanwhile, neutrophils express a heterodimeric receptor that has ligand binding specificity for the Arg-Gly-Asp sequence within many adhesive proteins including Fn, fibrinogen, vitronectin, von Willebrand's factor, and collagen type IV [24].

The importance of the determination of plasma Fn levels during Mg supplementation is stimulated by recent reports linking Mg and Fn. Several nuclear proteins have been shown to interact in an Mg-dependent fashion with a conditionally processed pre-mRNA derived from the Fn gene [25]. Mg alone has no effect on Fn binding to endothelial cells, but instead acts as an antagonist, suppressing the calcium-stimulated binding [26]. In addition, diltiazem prevents binding of Fn to cultured mesenchyme cells [27]. We observed a significant decline in the plasma Fn level in both the Mg and diltiazem groups during the reperfusion, when compared with saline-treated animals. Similar trends in the dynamics of Fn concentration have been observed after uncomplicated AMI in humans [23].

We also assessed the effects of Mg, diltiazem, and NPC 15669 on plasma Fn level in the absence of myocardial injury. While Mg and diltiazem had no effect on the plasma Fn levels in the animals free of ischemia, NPC 15669 therapy was associated with the dramatic increase of Fn levels. We found significant elevation in plasma Fn levels between the NPC- and saline-treated groups of animals with AMI. However, the difference was observed only at the very beginning of coronary artery occlusion. The relevance of the increased plasma Fn during the early occlusion phase in NPC 15669-treated animals remains uncertain.

After brief ischemia, we found a significant elevation in plasma Fn levels in the NPC-treated swine when compared with the saline-treated swine. Our findings of high Fn levels in swine that were treated with Mac-1 inhibitor are consistent with other studies linking Fn level with integrin expression. Fn was shown to play a key role for the TNF-alpha-mediated respiratory burst in neutrophils and could also modify the characteristics of the subsequent CD11b-CD18 integrins [28]. Suppression of intracellular Ca2+ mobilization and plasma Fn levels during ischemia-reperfusion can be explained by the blockage of Fn-integrin binding [29].

There are several limitations to the present study. The current study is primarily descriptive and, thus, cannot lead to direct statements of the involved mechanisms. Physiologic and pathologic significance of the observed plasma Fn changes during the different phases of ischemia-reperfusion is uncertain.

A swine model was chosen for several reasons. First, the anatomy of the swine is known to closely mimic the human coronary circulation, especially with regard to a relative absence of collateral flow [30]. Second, the Yorkshire swine's heart is large enough to produce a model of regional ischemia in which an uninvolved region can serve as an internal control. Third, swine and humans demonstrated similar platelet adhesion, aggregation, and release reactions [31,32]. Finally, swine lack significant myocardial xanthine oxidase activity, similar to humans [32]. It has been shown that xanthine oxidase activity is responsible for the endothelial activation, mediating platelet aggregation, and cyclic flow variations seen in stenosed and endothelium-injured coronary arteries [33]. We used an open-chested animal model. This design is not as physiologic as a closed-chest conscious porcine model and there are obvious concerns about the potential effects of filling pressure and neurohumoral activation on the plasma Fn. In summary, current data suggest that both prolonged and brief coronary artery occlusion followed by reperfusion resulted in the significant serial changes in the plasma Fn. Intracoronary low-dose Mg and diltiazem administration, like intravenous pretreatment with a novel Mac-1 inhibitor, are associated with the significant changes in the plasma Fn. To the best of our knowledge, this is the first observation that Mac-1 inhibition is associated with the elevated plasma levels of Fn in controls, and in the setting of mild MS or AMI. Supplemental Mg or diltiazem, and leumedins, may have a beneficial effect in patients with ischemic heart disease due to improved vasoreactivity and modulation of Fn production during myocardial ischemia-reperfusion.

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