Dipyridamole with Low-Dose Aspirin Augments the Infarct Size-Limiting Effects of Simvastatin

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Abstract

Purpose Statins protect against ischemia-reperfusion injury and limit myocardial infarct size (IS). This effect is dependent on increased generation of adenosine by ecto-5′ nucleotidase and downstream activation of cyclooxygenase-2 (COX2). Dipyridamole (DIP) augments the IS-limiting effects of statins by blocking the cellular reuptake of adenosine; whereas aspirin (ASA) attenuates the effect by inhibiting COX2. We studied the effect of acute administration of DIP, ASA and their combination on the IS-limiting effect of simvastatin (SIM).

Methods Rats received oral SIM (10 mg/kg/d) or vehicle for 3 days. Rats underwent 30 min of coronary artery occlusion and 4 h reperfusion. After 5 min of ischemia rats received i.v. DIP (5 mg/kg), ASA (20 mg/kg or 2 mg/kg) or DIP+ASA (2 mg/kg) or vehicle alone. Ischemia area at risk (AR) was assessed by blue dye and IS by TTC. Myocardial samples were analyzed for the activation of Akt, ERK 1/2, endothelial nitric oxide synthase (eNOS), and cyclic-AMP-response-element-binding-protein (CREB).

Results SIM limited IS. High- or low-dose ASA alone had no effect on IS. DIP alone or with low-dose ASA significantly reduced IS. Low-dose ASA did not attenuate the SIM effect, whereas high-dose ASA completely blocked the effect. The combination of DIP+low-dose ASA+SIM resulted in the smallest IS. Both SIM and DIP+low-dose ASA augmented Akt phosphorylation and their effect was additive. Both SIM and DIP+low-dose ASA augmented eNOS, ERK 1/2 and CREB phosphorylation.

Conclusions During acute myocardial ischemia, DIP alone or with low-dose ASA limits IS and does not attenuate the IS-limiting effect of SIM as high-dose ASA.

Key words Reperfusion injury · Statins · Adenosine · Dipyridamole · Aspirin

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Introduction

Dipyridamole (DIP) increases interstitial adenosine concentrations by blocking the cellular reuptake of adenosine [1, 2]. When administered intravenously at high doses (ie, 0.56 mg/kg over 4 min), DIP may exacerbate myocardial ischemia by diverting blood flow from diseased vessels with fixed narrowings to healthier branches undergoing vasodilatation [3]. However, when administered orally at low doses (ie, 300–400 mg/d), DIP has significant antiplatelet effects and causes minimal hemodynamic changes. In the clinical setting, DIP has been used to prevent cardiovascular and cerebrovascular events [2].

Recently, the Prevention Regimen for Effectively Avoiding Second Strokes (PRoFESS) study compared the effects of low-dose aspirin (ASA), combined with extended-release DIP (25 mg and 200 mg, respectively; twice daily), with the effects of clopidogrel (75 mg/d) on the secondary prevention of stroke [4]. In both treatment groups, the rates of recurrent stroke and of the combined endpoint—stroke, myocardial infarction (MI), and vascular death—were similar [4]. However, in patients with acute MI, the role of DIP (alone or in combination with low-dose ASA) is less clear.

When given to patients with stable angina or acute coronary syndromes, intracoronary DIP before percutaneous transluminal coronary angioplasty was reported to reduce the risk of abrupt vessel closure [5, 6]. Furthermore, intracoronary DIP reduced the incidence of adverse cardiovascular events in the first 48 h after balloon angioplasty of small coronary arteries [7]. Yoshida and colleagues [8] also reported that intracoronary DIP administered for acute anterior wall MI before primary percutaneous coronary intervention prevented reperfusion arrhythmia. However, in a study by Thompson and colleagues [9], 202 patients with ST-segment elevation myocardial infarction (STEMI) who were randomly assigned to receive either a continuous intravenous heparin infusion or oral DIP (100 mg, 3 times a day) and ASA (300 mg/d) 24 h after thrombolytic therapy had similar outcomes. Seven to 10 days after treatment, no differences were observed in clinical events or the rates of patency of the infarct-related artery [9].

The American College of Cardiology (ACC)/American Heart Association (AHA) guidelines for the management of patients with STEMI do not recommend the use of DIP as an antiplatelet agent. According to these guidelines, the combination of low-dose ASA and extended-release DIP is reserved for STEMI patients who have ischemic stroke but do not undergo percutaneous coronary intervention and do not have a cardiac source of embolism or a surgically important carotid stenosis [10–12]. The possibility that DIP could cause coronary steal and augment ischemia in the setting of acute MI may be the reason for these guidelines.

Numerous studies have shown that the hydroxymethyl glutaryl coenzyme A reductase inhibitors (statins) reduce myocardial infarct size [13–18]. The use of statins for patients at high risk for cardiovascular events and for those presenting with acute myocardial infarction is recommended by the guidelines. Previously, we showed that when given orally, DIP augmented the effects of atorvastatin by further reducing the myocardial infarct size (IS) in rats [13]. Statins activate ecto-5′-nucleotidase, which generates adenosine [14]. Inhibition of adenosine receptors not only abrogates the IS-limiting effect of statins [13, 14], but prevents statins from inducing the phosphorylation of the prosurvival mediators ERK 1/2, Akt, and endothelial nitric oxide synthase (eNOS) [15]. The protective, IS-limiting effects of statins are also dependent on the activation of cyclooxygenase-2 (COX2) [16, 17], which can be attenuated by ASA, a nonspecific cyclooxygenase inhibitor, in a dose-dependent manner [18].

In our study, we investigated the effects of high- or low-dose ASA and DIP on infarct size, when given alone or in combination with simvastatin (SIM), a hydroxymethyl glutaryl coenzyme A reductase inhibitor (statin), during myocardial ischemia in rats.

Materials and methods

Animal care

Experiments were performed in male Sprague-Dawley rats. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the United State National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Materials

DIP was provided by Boehringer Ingelheim GmbH (Germany). ASA was obtained from Sigma (St. Louis, MO) and SIM from Merck (Whitehouse Station, NJ). Anti-Akt, anti-Thr308 P-Akt, and anti-Ser-473 P-Akt antibodies were obtained from R&D Systems (Minneapolis, MN). Anti-eNOS, anti-Ser-1177 P-eNOS, anti-ERK 1/2, anti-Thr-202/Tyr-204 P-ERK 1/2, anti-CREB, and anti-Ser-133 P-CREB antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Monoclonal anti-β-actin antibodies were obtained from Sigma.

Treatment

Rats received either simvastatin (10 mg/kg/d) or vehicle (water) by means of oral gavage once daily for 3 days. On the fourth day, the rats were anesthetized with an intraperitoneal injection of ketamine (60 mg/kg) and xylazine (6 mg/kg) and were intubated and ventilated (FIO$_2$=30%). The rectal temperature of the rats was monitored, and their body temperature was maintained between 36.7° and 37.3°C throughout the experiment. After the left carotid artery was cannulated, the chest was opened through the fourth intercostal space. The left coronary artery was encircled with a suture and ligated for 30 min. Five minutes after the start of ischemia, 1 of the following agents was infused intravenously for 120 min: vehicle (control); high-dose ASA (20 mg/kg, ASA20); low-dose ASA (2 mg/kg, ASA2); DIP alone (5 mg/kg); or a

combination of ASA2 and DIP. Isofluorane (1%–2.5%, titrated to effect) was given to the rats after the start of ischemia to maintain anesthesia. After 30 min of ischemia, the snare was released and myocardial reperfusion was verified by a change in the color of the myocardium. Subcutaneous buprenorphine (0.1 mg/kg) was administered, the chest was closed. At the end of the infusion (25 min during myocardial ischemia and the first 95 min of reperfusion), the rats were allowed to recover from anesthesia at the end of the infusion. Four hours after reperfusion (IS protocol) and 95 min after reperfusion (immunobloting), the rats were re-anesthetized, and the coronary artery was reoccluded; 1.5 ml of Evans blue dye (3%) was injected into the right ventricle, and the rats were euthanized while under deep anesthesia [13, 16, 17]. The heart rate (HR) and mean blood pressure (MBP) were recorded immediately before coronary artery occlusion, after 20 min of ischemia, and after 20 min of reperfusion.

The prespecified exclusion criteria included a lack of signs of ischemia during coronary artery ligation, lack of signs of reperfusion after release of the snare, prolonged ventricular arrhythmia with hypotension, and an area at risk measuring ≤10% of the left ventricular (LV) weight.

Determination of area at risk and infarct size

The rat hearts were excised, and the left ventricle was sliced transversely into 6 sections. The slices were incubated for 10 min at 37°C in 1% buffered (pH=7.4) 2,3,5-triphenyl-tetrazolium-chloride (TTC), and were fixed in a 10% formaldehyde solution. The sections were photographed to identify the ischemic area at risk (AR)(not colored by the blue dye), the infarct size (IS)(unstained by TTC), and the nonischemic zones (colored by blue dye). In each slice, the AR and IS were determined by means of planimetry, converted into percentages of the whole for each slice, and multiplied by the weight of the slice. The results were summed to obtain the weight of the myocardial AR and IS [13, 16, 17].

Immunoblotting

Rats in the control group were treated with water, by means of oral gavage for 3 days, and were not subjected to the ischemia-reperfusion protocol. The other groups were treated as above and exposed to 30 min of ischemia and 95 min of reperfusion. There were 4 animals in each group. Myocardial samples from the border of the previously identified ischemic AR zone of the LV wall of treated rats and myocardial samples of control rats were homogenized in lysis buffer (25 mmol Tris-HCl [pH=7.4], 0.5 mmol EDTA, 0.5 mmol EGTA, 1 mmol phenylmethylsulfonyl fluoride, 1 mmol dithiothreitol, 25 mmol NaF, 1 mmol Na_3VO_4, 1% Triton X-100, 2% sodium dodecyl sulfate [SDS], and 1% protease inhibitor cocktail). The lysate was centrifuged at 10,000 × g for 15 min at 4°C, and the supernatants were collected. Protein (50 μg) was fractionated by SDS polyacrylamide gel electrophoresis (PAGE; 4%–20%) and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA), which were incubated overnight at 4°C with primary antibodies. Bound antibodies were detected by using a chemiluminescent substrate (NEN Life Science Products, Boston, MA). The protein signals were quantified with an image-scanning densitometer, and the strength of each protein signal was normalized to the corresponding β-actin signal. The data are expressed as a percentage of the expression in the control group.

Statistical analysis

Unless otherwise noted, the data are presented as the mean ± standard error of the mean. Body weight, left ventricular weight, the size of the area at risk and infarct size, and protein expression were compared by using analysis of variance (ANOVA) with Sidak corrections for multiple comparisons. The differences in HR and MBP were compared by using two-way repeated-measures (treatment × time) ANOVA with Holm-Sidak multiple comparison procedures. $P<0.05$ was considered statistically significant.

Results

IS protocol

A hundred rats began the protocol, 4 rats died during the induction of anesthesia (1 in the SIM group, 1 in the ASA20 group, 1 in the SIM+ASA20, and 1 in the ASA2+DIP group) and 2 during surgery (1 in the ASA20 and 1 in the SIM+ASA20 group). A total of 94 rats were included.

Body weight, left ventricular weight, and area at risk sizes were comparable among groups (Table 1). The IS, expressed as a percentage of the left ventricular weight (Table 1) or as a percentage of the AR (Table 1; Fig. 1), was significantly smaller in the SIM, ASA2+DIP, DIP, SIM+ASA2, and SIM+ASA2+DIP groups than in the control group. The IS in the ASA2, ASA20, and SIM+ASA20 groups was not significantly different from that in the control group (Table 1; Fig. 1). Both the 3-day pretreatment with SIM alone and the intravenous administration of DIP during occlusion reduced the IS. However, low- or high-dose ASA alone (2 mg/kg or 20 mg/kg, respectively) administered during occlusion had no effect on IS. In rats pretreated with SIM, however, high-dose ASA blocked the protective IS-limiting effect of SIM. Low-dose ASA did not attenuate the effects of SIM or DIP. The SIM+ASA2+DIP

Table 1 Body weight, left ventricular weight, area at risk, and infarct size of control and treated rats
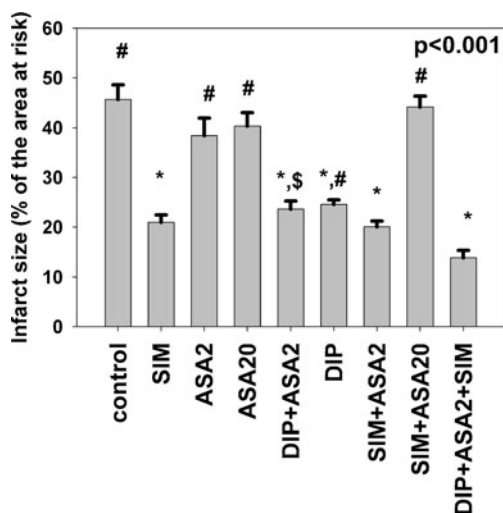
Treatment group	BW (g)	LV weight (mg)	Area at risk (% of LV weight)	Infarct size (% of LV weight)	Infarct size (% of area at risk)
Control (n=10)	259±5	1105±7	29.3±0.8	13.5±1.1	45.7±2.9
SIM (n=11)	259±2	1110±2	30.9±1.1	6.4±0.3	21.0±1.5
ASA2 (n=10)	257±3	1110±4	28.4±0.7	10.9±1.1	38.4±3.5
ASA20 (n=10)	258±3	1112±4	28.5±0.8	11.6±0.9	40.3±2.7
ASA2+DIP (n=10)	259±3	1115±4	27.4±0.8	6.5±0.5	23.6±1.7
DIP (n=10)	258±2	1116±3	29.0±0.9	7.1±0.4	24.6±0.9
SIM+ASA2 (n=13)	259±2	1114±4	27.2±0.9	5.4±0.3	20.0±1.2
SIM+ASA20 (n=10)	259±1	1115±3	28.2±0.7	12.4±0.5	44.2±2.1
SIM+ASA2+DIP(n=10)	259±1	1115±4	28.1±0.8	3.8±0.3	13.8±1.5
P value	0.999	0.570	0.138	<0.001	<0.001

BW body weight; *LV* left ventricular; *SIM* simvastatin; *DIP* dipyridamole; *ASA* aspirin

group showed the smallest IS (Fig. 1), although the differences between the IS of this group and the IS of the SIM+ASA2 or the SIM-alone groups did not reach statistical significance. The difference between the IS of the SIM+ASA2+DIP and ASA2+DIP groups was of borderline significance (P=0.061; Fig. 1).

The HR was unaffected by the different treatments (P= 0.062). Although significant changes in HR were observed in all rats from the time of preocclusion to 20 min after reperfusion (P<0.001), the interaction between treatment and time was not significant (P=0.166; Fig. 2a). The MBP significantly decreased from the time of preocclusion to 20 min after reperfusion (P<0.001; Fig. 2b), but the difference among the control and treatment groups was

Fig. 1 Myocardial infarct size (*IS*) shown as a percentage of the area at risk (*AR*) in control and treatment groups. Data are presented as the mean ± standard error of the mean. Overall, a significant difference was observed in infarct size among different treatment groups (P< 0.001). * P<0.001 versus control; # P<0.001 versus DIP+ASA2 +SIM; $ P=0.061 versus DIP+ASA2+SIM

not significant (P=0.062). However, with respect to MBP, the interaction between treatment and time was significant (P=0.001). During coronary occlusion, the MBP was significantly lower in the SIM+ASA2+DIP group (98.6± 9.4 mmHg) than in the control group (112.0±0.3 mmHg), the DIP-alone group (111.7±2.4 mmHg), and the SIM +ASA2 group (115.5±2.2 mmHg) but not in the ASA2 +DIP group (106.4±2.3 mmHg). In addition, during coronary occlusion, the MBP was significantly higher in the SIM+ASA2 group (115.5±2.2 mmHg) than in the SIM +ASA20 group (104.4±0.9 mmHg) but not in the ASA20-only group (108.0±2.3 mmHg). These differences are not consistent with any of the drugs' known effects and were most likely random. No other significant changes were detected from baseline (preocclusion) to the time of reperfusion.

Immunoblotting

There were 4 animals in each group. The total Akt and total eNOS protein levels were comparable among the treatment and control groups (Fig. 3). Ischemia-reperfusion alone increased myocardial levels of Ser-473 P-Akt and Thr-308 P-Akt compared to controls not subjected to ischemia-reperfusion (Fig. 3). SIM and ASA2+DIP increased Ser-473 P-Akt and Thr-308 P-Akt levels, whereas ASA20 had no significant effect. For the SIM+ASA20 group, ASA20 did not block the SIM-induced increase in P-Akt levels (Fig. 3), despite blocking the IS-limiting effects of SIM (Fig. 1). The highest levels of Ser-473 P-Akt and Thr-308 P-Akt were seen in the SIM+ASA2+DIP group (Fig. 3).

Ischemia-reperfusion alone caused a mild increase in Ser-1177 P-eNOS levels (Fig. 3). SIM and ASA2+DIP increased P-eNOS levels. ASA20 had no significant effect on P-eNOS levels, and in the SIM+ASA20 group, ASA20 did not block the effect of SIM. No significant differences
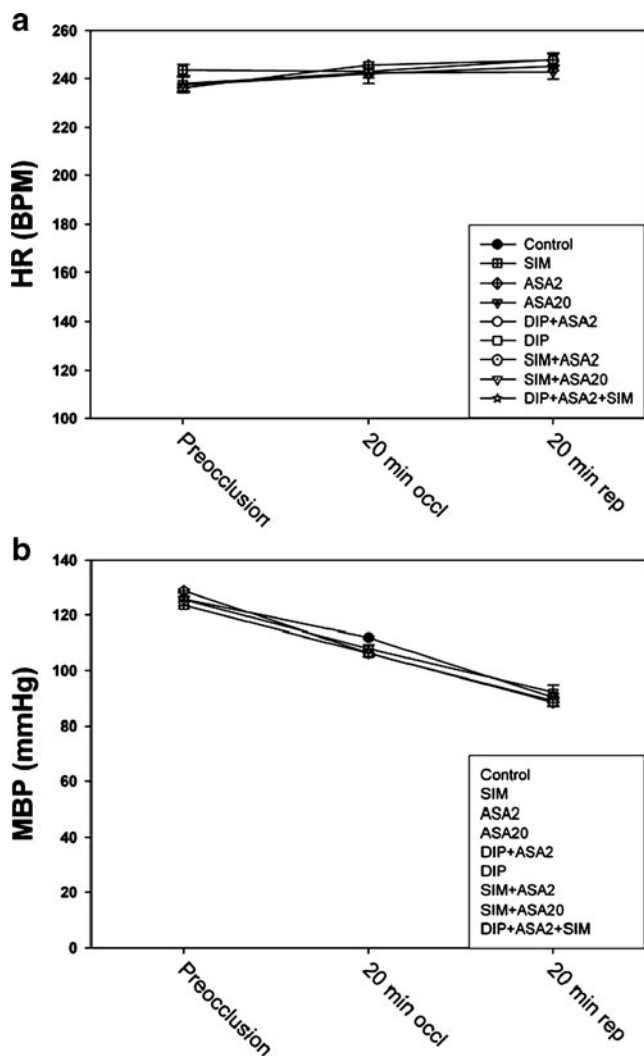
Fig. 2 Heart rate (*HR*, shown in beats per minute) and mean blood pressure (*MBP*, shown in mmHg) from pre-occlusion to 20 min after the start of reperfusion. **a** The different treatments did not significantly affect HR ($P=0.062$). HR changed significantly over time ($P<0.001$), but the interaction between treatment and time was not statistically significant ($P=0.166$). **b** MBP significantly decreased over time ($P<0.001$). Overall, no significant differences were observed in MBP among the treatment groups ($P=0.062$), although there was a significant interaction between treatment and time ($P=0.001$)

were observed in Ser-1177 P-eNOS levels among the SIM, SIM+ASA20, ASA2+DIP, and SIM+ASA2+DIP groups (Fig. 3).

As shown in Fig. 4, the protein levels of total ERK 1/2 or cyclic AMP response element binding protein (CREB) were comparable among the treatment and control groups. Ischemia-reperfusion alone increased myocardial levels of P-ERK 1/2. ASA20 had no significant effect, whereas SIM alone or with ASA20 augmented myocardial levels of P-ERK 1/2. DIP+ASA2 increased P-ERK 1/2 levels more than did SIM ($P=0.006$) or SIM+ASA20 ($P=0.024$). P-ERK 1/2 levels were significantly higher in the DIP+ASA2+SIM

group than those in all other groups, except the DIP+ASA2 group ($P=0.916$).

Ischemia-reperfusion alone increased P-CREB levels (Fig. 4). ASA20 did not alter the effect of ischemia-reperfusion on P-CREB. However, SIM, SIM+ASA20, DIP+ASA2, and DIP+ASA2+SIM increased myocardial levels of P-CREB equally (Fig. 4).

Discussion

In our study, we showed that the intravenous infusion of DIP (5 mg/kg over 2 h), started during myocardial ischemia and continued during the first 95 min of reperfusion, significantly reduced IS to an extent comparable with 3-day SIM pretreatment, without causing significant hemodynamic changes. Furthermore, when the intravenous infusion of DIP and low-dose ASA was combined with SIM pretreatment, the IS was reduced to an even greater extent than with SIM or DIP alone. Low-dose ASA did not affect the IS-limiting effects of SIM, but high-dose ASA completely blocked the IS-limiting effects of SIM.

Previously, we have shown that pretreating rats with orally administered DIP (6 mg/kg/d) and low-dose atorvastatin (2 mg/kg/d) limited IS in a synergistic manner [13]. When administered alone, however, neither low-dose atorvastatin nor DIP affected the infarct size. Low-dose atorvastatin and DIP each caused a small increase in myocardial adenosine levels, although when these agents were combined, adenosine levels were significantly increased. Furthermore, only the combination of low-dose atorvastatin and DIP increased myocardial Ser-473 P-Akt and Ser-1177 P-eNOS levels [13]. Aminophylline, a nonselective adenosine receptor blocker, abrogated the IS-limiting effect of DIP and atorvastatin, suggesting that the protective effects of both drugs are mediated by the activation of adenosine receptors.

In our experiments, we used a rate of infusion of DIP (0.042 mg/kg/min) significantly lower than the rate used to induce ischemia during pharmacologic stress tests (0.14 mg/kg/min); however, we used an absolute dose that is much higher (5 mg/kg versus 0.56 mg/kg) [3]. DIP blocks the cellular reuptake of adenosine, thus increasing interstitial adenosine levels [1, 2]. DIP causes vasodilation of blood vessels and has antiplatelet effects [1, 2]. In addition, Harker and Kadatz have suggested that DIP is a cAMP phosphodiesterase inhibitor and that it increases the biosynthesis of prostacyclin (PGI_2) [1].

The IS-limiting effects of statins are dependent on the activation of ecto-5-nucleotidase, which generates adenosine and phosphatidylinositol-3-kinase that promotes the phosphorylation of the pro-survival kinase Akt [14]. The inhibition of adenosine receptors abrogates the IS-limiting
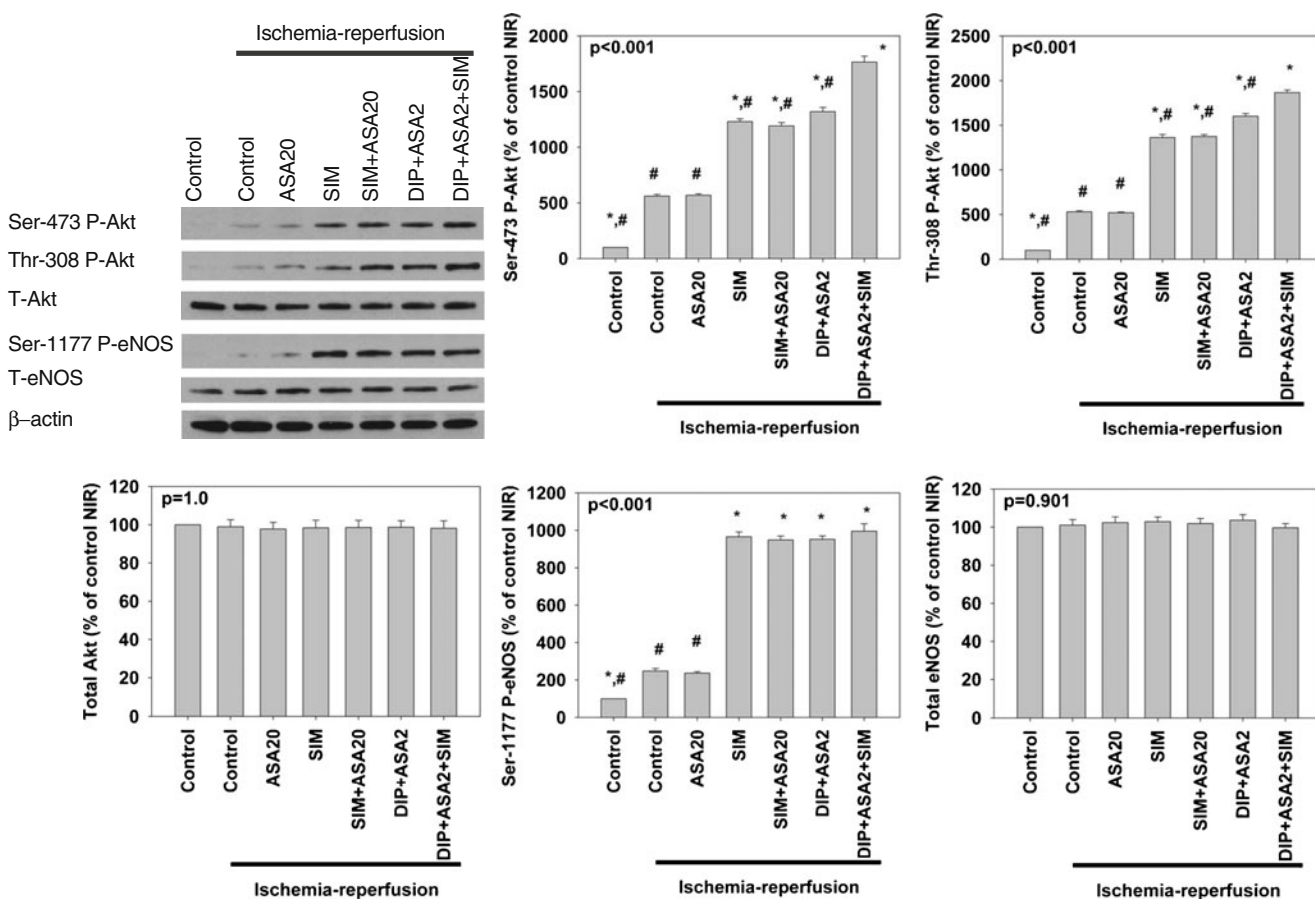
Fig. 3 Representative immunoblots and densitometric analyses of Ser-473 P-Akt, Thr-308 P-Akt, total Akt, Ser-1177 P-eNOS, and total eNOS in the border zone of hearts exposed to the ischemia-reprefusion protocol. The myocardium of rats that were not exposed to ischemia-reperfusion and received vehicle served as the control. β-actin was used as a loading control. * $P<0.001$ versus ischemia-reperfusion control; # $P<0.001$ versus DIP+ASA2+SIM

effects of statins [13, 14] and prevents statins from inducing the phosphorylation of the prosurvival mediators Erk 1/2, Akt, and eNOS [15]. We have previously shown that the adenosine receptors A_1, A_{2A}, and A_{2B} are involved in the statin-induced activation of Akt and eNOS [15]. Activation of the adenosine receptors A_{2A} and A_{2B} leads to an increase in intracellular cAMP and the subsequent activation of protein kinase A (PKA), in addition to the activation of other prosurvival kinases, including ERK 1/2 [19]. Both PKA [20, 21] and its downstream effector, phosphorylated CREB (P-CREB) [22, 23], are involved in myocardial protection against ischemia-reperfusion-related injury.

In our study, SIM and DIP+ASA2 increased myocardial levels of P-CREB. A major nuclear transcription factor, CREB transduces signals from cAMP to activate gene transcription [24]. CREB is activated by phosphorylation at Ser-133 by PKA [24, 25], which can also be mediated by Akt [24, 26], the MEK/MAPK/p90rsk pathway [27], $Ca^{2+}/$ calmodulin-dependent protein kinase II [27], protein kinase Cε [28], protein kinase D [29], ERK 1/2, and mitogen and stress-activated protein kinase-1 (MSK-1) [22]. CREB

phosphorylation induces the translocation of CREB from the cytoplasm to the nucleus where it regulates the transcription of target genes [24]. CREB also binds a cAMP-response element site in the promoter region of genes such as cytochrome C and Bcl-xl, which CREB has been shown to positively regulate [30]. CREB is involved in ischemic preconditioning [22, 23].

Interestingly, despite the fact that ASA20 blocked the IS-limiting effect of SIM, it did not attenuate the phosphorylation of CREB, suggesting that COX2 functions downstream of CREB activation. Moreover, despite a smaller IS in the DIP+ASA2+SIM group, comparable myocardial levels of P-CREB were observed in the SIM, SIM+ASA20, and DIP+ASA2 groups, suggesting that other signaling pathways are involved in limiting IS.

In addition to activating the cAMP, PKA, and CREB pathways, SIM and DIP+ASA2 activated 2 prosurvival kinases–Akt and ERK 1/2— that are known to mediate protection against ischemia-reperfusion and reperfusion injury [31, 32]. Although high-dose ASA attenuated the IS-limiting effect of SIM, it did not block the effect of SIM

on Akt and ERK 1/2 phosphorylation (Figs. 3 and 4), because Akt and ERK 1/2 activation are upstream of COX2 activation [15]. The effects of DIP+ASA2 and SIM on the phosphorylation of Akt were additive when all 3 treatments were combined; this finding paralleled the effect of these agents on myocardial IS. However, it is interesting that DIP+ASA2 increased P-ERK 1/2 levels more than did SIM or SIM+ASA20 (Fig. 4) and that the effect of DIP+ASA2 on ERK 1/2 phosphorylation was comparable to that of SIM+DIP+ASA2, suggesting that there was no additive effect.

As previously described, pretreatment with statins augmented eNOS phosphorylation [13, 14, 17]. eNOS activation is essential for mediating the protective effects of statins, and it is upstream of COX2 activation [33]. In the present study we are showing that DIP+low-dose ASA infusion during ischemia and early reperfusion had equal effect on eNOS phosphorylation as 3-day pretreatment with SIM. However, the combination of SIM with DIP+ASA2 did not result in additive effect on eNOS phosphorylation in myocardium subjected to ischemia-reperfusion. As expected, high-dose ASA did not block the effect of pretreatment with SIM, as COX2 is downstream of eNOS activation [33].

COX2 activation is essential for the IS-limiting effects of statins [17, 18]. We have previously shown that ASA dose dependently (5, 10 and 20 mg/kg) attenuated COX2 activity and that COX2 activity inversely correlated with IS [18]. We have also shown that COX2 inhibition abrogates the IS limiting effects of atorvastatin without affecting the upregulation of eNOS phosphorylation and iNOS expression by atorvastatin [17].

We found that ASA20, but not ASA2 attenuated the IS limiting effects of SIM. In the clinical setting, it is recommended that patients with acute ST elevation myocardial infarction will receive chewed aspirin at 160–325 mg/kg [18]. In Europe an intravenous dose of 250–500 mg is often used. For a 70 kg patient this represents up to 7.1 mg/kg. Doses of medications given to rats and mice are usually much higher than those used in the clinical setting. We have previously shown that intravenous aspirin (10 and 20 mg/
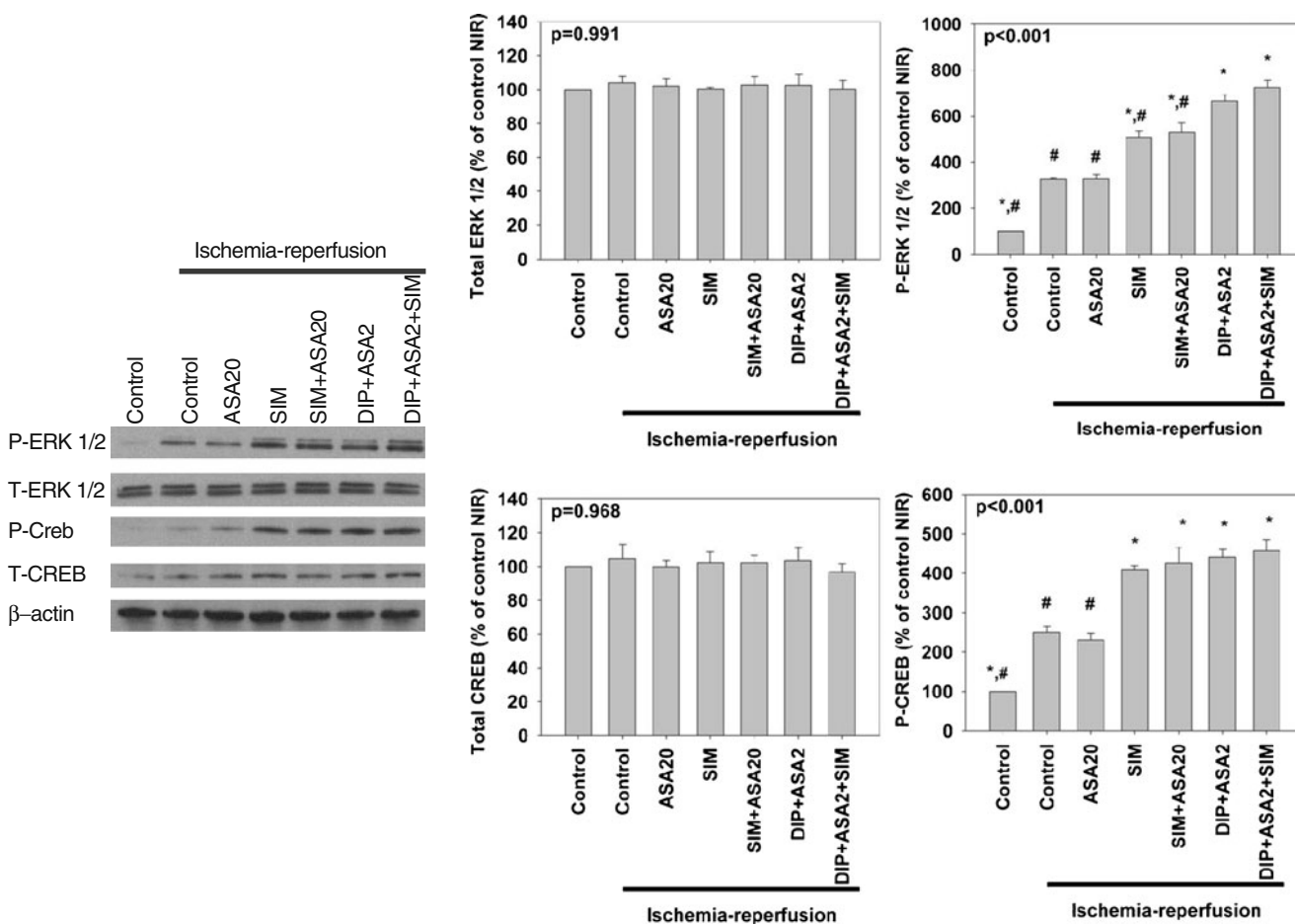
Fig. 4 Representative immunoblots and densitometric analyses of total ERK 1/2, Thr 202/ Tyr-204 P-ERK 1/2, total CREB, and Ser-133 P-CREB in the border zone of hearts exposed to ischemia-reperfusion. The myocardium of rats that were not exposed to ischemia-reperfusion and received vehicle served as the control. β-actin was used as a loading control. * $P < 0.001$ versus ischemia-reperfusion control; # $P < 0.001$ versus DIP+ASA2+SIM

kg), administered before reperfusion, attenuates the infarct size limiting effects of atorvastatin [18]. At 5 mg/kg there is attenuation, but it does not reach statistical significance [18]. Further studies are needed to assess whether such adverse interaction occurs in the clinical setting.

Limitations: in the present study we did not assess the effect of DIP alone without low-dose ASA on the expression of the prosurvival kinases; however, ASA2 did not attenuate the IS-limiting effects of DIP (Fig. 1). Furthermore, we have previously shown that the inhibitory effect of ASA on the IS-limiting effects of atorvastatin is dose dependent and is only minimal at 5 mg/kg [18]. ASA alone, even at the higher dose, does not affect myocardial IS [18]. The results of our two studies are comparable, showing that at 2 mg/kg, ASA does not attenuate the protective effect of SIM (Fig. 1).

In our model, myocardial infarction is induced in rats by mechanically compressing the artery, rather than by causing the spontaneous formation of a blood clot on a ruptured coronary plaque. In the clinical setting, the inhibition of platelet activity has a significant role in the treatment of acute myocardial infarction [10, 11]. Although DIP+ASA2 have antiplatelet properties, their effects should be compared to those of ASA combined with thienopyridines, which are currently the first-choice agents for inhibiting platelet aggregation per ACC/AHA guidelines. Therefore, studies are warranted that would compare the effects of high-dose ASA with thienopyridine versus low-dose ASA+DIP+thienopyridine; or alternatively, low-dose ASA+DIP versus thienopyridine with ASA in patients receiving statin agents.

In conclusion, when started after coronary artery occlusion and continued during early reperfusion, intravenous infusion of DIP+ASA2 appeared to be safe and was not associated with significant hemodynamic changes. Furthermore, DIP+ASA2 was associated with a significant reduction in the myocardial IS and with activation of the prosurvival kinases Akt and ERK 1/2, CREB, and eNOS. Thus, the combination of DIP and low-dose ASA should be considered as an alternative to the higher dose ASA currently used, especially in patients receiving statins. Further studies are needed to verify if such interactions occur in the clinical setting.

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