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Short communication

In vivo myocardial infarct size reduction by a caspase inhibitor administered after the onset of ischemia

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Abstract

The aim of this study was to determine the effect of different administration protocols on the cardioprotective efficacy of the non-selective, irreversible caspase inhibitors *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) and bocaspartyl-(OMe)-fluoromethylketone (BocD.fmk) in a rat in vivo ischemia and reperfusion paradigm. Hearts were made ischemic for 45 min and reperfused for 180 min. Under these conditions, it was determined that zVAD.fmk was cardioprotective when administered before or after the onset of ischemia, whereas BocD.fmk was efficacious only when administered before the onset of ischemia. This is the first report of in vivo cardioprotection by a caspase inhibitor when administered after the onset of ischemia.

Keywords: Apoptosis; Cardiac ischemia; Infarct; Caspase inhibitor; Reperfusion injury

1. Introduction

Recognition that neutrophils are activated by and infiltrate into damaged tissue during post-ischemic reperfusion, and thus play an important role in in vivo cardiac reperfusion injury, (Jordan et al., 1999; Romson et al., 1983) and furthermore, that administration of superoxide dismutase/catalase reduced infarct size (Jolly et al., 1984), provided pivotal evidence in support of the theory that cardiac cell death during reperfusion could be pharmacologically reduced. Neutrophil-mediated myocyte death is necrotic/oncotic in nature (Majno and Joris, 1995). However, cardiac myocytes also die by apoptosis (Bialik et al., 1997; Black et al., 1998; Fliss and Gattinger, 1996; Gottlieb et al., 1994; Kajstura et al., 1996). Apoptosis involves an intrinsic proteolytic cascade (Kerr et al., 1972; McConkey et al., 1996), within which the caspase family of enzymes are critical (Nicholson and Thornberry, 1997), i.e., activated caspase-3 has been linked to apoptotic myocyte death in rat (Black et al., 1998). Pharmacologically, apoptotic myocyte death and infarct size are reduced by caspase inhibitors (Holly et al., 1999; Okamura et al., 2000; Yaoita et al., 1998). However, in all studies to date, caspase inhibitors have been administered prior to the onset of ischemia. Although apoptosis is initiated by ischemia, reperfusion was either required for the cell death to occur (Freude et al., 2000), or to accelerate apoptotic death (Fliss and Gattinger, 1996). It has not been reported that administration of a caspase inhibitor immediately before the onset of reperfusion may reduce infarct size. This would be important to demonstrate if caspase inhibitors are to be considered as potential adjunctive therapy to coronary artery thrombolysis or percutaneous transluminal coronary angioplasty. Thus, the aim of the current study was to determine pharmacologic efficacy of caspase inhibitors administered after the onset of ischemia. For comparative purposes, we determined the cardioprotective efficacy of two non-selective, irreversible caspase inhibitors, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk) and bocaspartyl-(OMe)-fluoromethylketone (BocD.fmk), on infarct size with the inhibitors administered before the onset of ischemia or after ischemia onset.

2. Materials and methods

All experiments were approved by the Animal Care Committee at Merck Frosst in accordance with guidelines

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established by the Canadian Council on Animal Care. Male Sprague Dawley rats (300–400 g, acclimated for a minimum of 48 h) were used.

2.1. Surgical preparation of animals

Rats were anesthetized with intraperitoneal administration of sodium pentobarbital (65 mg/kg). Heart rate and aortic pressure were monitored by left carotid artery cannulation. The aortic cannula was interfaced with a pressure transducer (Triton Technology, San Diego, CA) and physiologic recorder (System 6, Triton Technology). The left jugular vein was isolated for administration of zVAD.fmk, BocD.fmk or vehicle (2% dimethylsulfoxide in 0.9% NaCl). The left coronary artery was occluded 2–3 mm from its origin with a 4.0 suture. After placement of the suture, the thoracotomy was closed and opened only to effect occlusion and reperfusion of the artery. A Lead II electrocardiograph (ECG) was continuously monitored.

2.2. Experimental protocol

After a baseline period of 30 min, the left coronary artery was occluded for 45 min, and reperfused for 3 h. In the pre-ischemia dosing protocol, zVAD.fmk (Enzyme Systems Biochemicals, CA) or BocD.fmk (Enzyme Systems Biochemicals) were administered as an intravenous (i.v.) bolus of 500 μg/rat (in 0.5 ml vehicle), 5 min before the onset of ischemia, and this dose and volume were administered again at the onset of reperfusion. Additionally, a continuous infusion of 5 μg/min × 180 min (5 μl/min) was initiated immediately after the first bolus dose. The total dose of zVAD.fmk or BocD.fmk was 2 mg. Control animals received equivalent vehicle alone (2% dimethylsulfoxide in 0.9% NaCl). In the post-ischemia dosing protocol, zVAD.fmk or BocD.fmk (Enzyme Systems Biochemicals) were administered as a continuous infusion of 42 μg/min × 120 min starting 10 min before the onset of reperfusion. The total dose of zVAD.fmk or BocD.fmk in the post-ischemia dosing protocol was 5 mg. Control animals received the vehicle alone (2% dimethylsulfoxide in 0.9% NaCl).

2.3. Infarct size determination

Area at risk of infarction and infarct size were determined in the excised heart using a dual staining technique: 1.5% w/v triphenyltetrazolium chloride (37°C) was infused via the aorta and the reaction continued for 2–3 min. The area at risk of infarct was demarcated by occluding the coronary artery and infusing a solution of 0.25% w/v Evan's blue. The stained heart was cut transversely into four slices of equal thickness, scanned using a color scanner (ScanMaker E3, Microtek, CA) interfaced with a computer (Macintosh, CA) running Color It! software (V. 3.0.3 PowerPC, MicroFrontier, IA). Infarct size and area at risk were quantified using a software program (MacDraft 4.2.1 PPC, Innovative Data Design, CA).

2.4. Statistical analysis

For comparisons between control, zVAD.fmk- and BocD.fmk-treated groups, data were analyzed using analysis of variance (ANOVA) (StatView 4.5, Abacus Concepts, Berkeley, CA). Where ANOVA detected a statistically significant ($P < 0.05$) difference, data were analyzed by a Bonferroni test to determine where statistical differences existed between the groups. All values are mean ± standard error of the mean (S.E.M.).

3. Results

Hemodynamic data were recorded continuously during the baseline, ischemic and reperfusion periods of the experiment. Heart rate and mean arterial pressure during the baseline, ischemic or reperfusion periods in the three different groups were not different in either dosing protocol. Ischemia-reduced ventricular function in all animals to a similar (not statistically different) extent; this was manifest as a reduction in mean arterial pressure (starting at the onset of ischemia). These data are not shown for brevity. Similarly, heart rates were not different between the groups at any time during the study, in either dosing protocol (data not shown for brevity). The ECG of all rats included in this study demonstrated significant and sustained ST segment elevation after the onset of ischemia. The ECG changed during reperfusion as Q waves developed: there was no effect of either zVAD.fmk or BocD.fmk (pre-ischemia or post-ischemia dosing protocols) on the development of the Q wave (ECG waveforms not shown for brevity).

The area of left ventricle at risk of infarct was not different between the control, zVAD.fmk or BocD.fmk groups in either the pre-ischemia or post-ischemia dosing protocols. In the pre-ischemia dosing protocol, the areas at risk were 42 ± 2.9, 43.8 ± 3.2, 37.8 ± 3.9 in the control, zVAD.fmk and BocD.fmk treated groups, respectively. In the post-ischemia dosing protocol, the areas at risk were 44.3 ± 6.5, 54.0 ± 2.1, 40.3 ± 2.3 in the control, zVAD.fmk and BocD.fmk treated groups, respectively.

The effect of the caspase inhibitors on infarct size is shown in Fig. 1. As shown in Fig. 1A, infarct size was significantly ($P < 0.05$) reduced in both the zVAD.fmk- and BocD.fmk-treated groups. The infarct size data of Fig. 1B indicate that when administered well after the onset of ischemia, and just before reperfusion, zVAD.fmk significantly ($P < 0.05$) protected the heart from ischemic/reperfusion injury. Post-ischemia dosing with BocD.fmk, however, did not protect the heart. Indeed, in rats adminis-

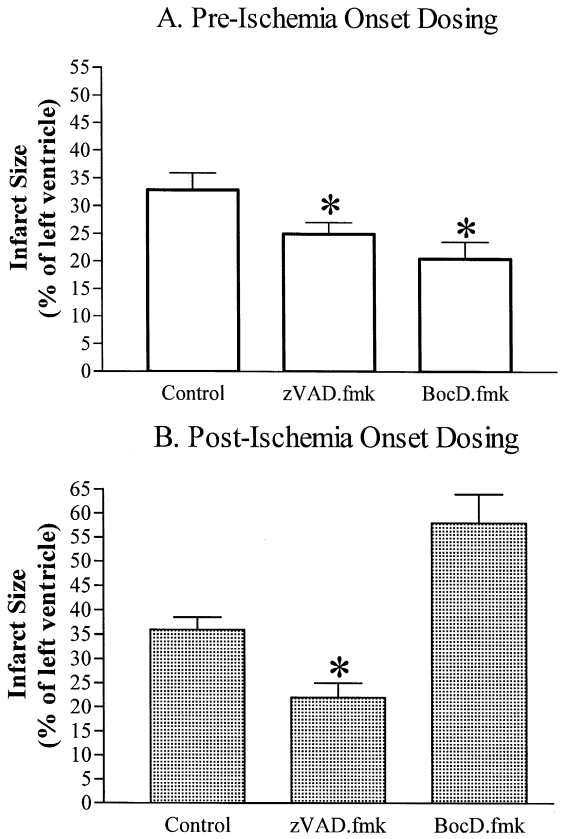

Fig. 1. The effect of non-selective caspase inhibitors zVAD.fmk and BocD.fmk on infarct size in the in vivo rat model of ischemia (45 min) and reperfusion (3 h). (A) Pre-ischemia dosing protocol and infarct size expressed as a percentage of the left ventricle in the control ($n = 11$), zVAD.fmk-treated ($n = 9$) and BocD.fmk-treated ($n = 8$). (B) Post-ischemia dosing protocol and infarct size expressed as a percentage of the left ventricle in the control ($n = 7$), zVAD.fmk-treated ($n = 10$) and BocD.fmk-treated ($n = 3$). * = Significantly different from control ($p < 0.05$).

tered with BocD.fmk, infarct size appeared to be exacerbated; however, the effect was not statistically significant compared to the control group.

4. Discussion

In this study, the non-selective, irreversible caspase inhibitors, zVAD.fmk and BocD.fmk were shown to reduce infarct size in a rat in vivo ischemia and reperfusion model when administered before the onset of ischemia. These results confirm previous findings whereby i.v. administration of zVAD.fmk before the onset of ischemia reduced infarct size (Holly et al., 1999; Okamura et al., 2000; Yaoita et al., 1998), and furthermore show that a structurally distinct caspase inhibitor (BocD.fmk) can also protect myocardial tissue when administered before the onset of ischemia. More importantly, and in contrast to all previous publications of cardioprotective efficacy of caspase inhibitors (Holly et al., 1999; Okamura et al., 2000; Yaoita et al., 1998), our results show that zVAD.fmk reduces infarct size when administered well after the onset of ischemia, immediately prior to the start of reperfusion. Interestingly, BocD.fmk, did not reduce infarct size when administered after the onset of ischemia. The reason for the inefficacy of BocD.fmk when administered after the onset of ischemia is not known, however, differences in the pharmacokinetic profile or other factors may account for the failure of BocD.fmk in the post-ischemia onset study. The degree of infarct size reduction by zVAD.fmk and BocD.fmk was similar to the degree of protection previously reported (Holly et al., 1999; Okamura et al., 2000; Yaoita et al., 1998). The extent of tissue protection by peptide-based caspase (cysteine protease) inhibitors is not as profound as the degree of protection that has been shown in rat heart affected by ischemic preconditioning (Piot et al., 1997). However, it is noteworthy that cardioprotection afforded by zVAD.fmk or BocD.fmk in the current study occurred independent of any hemodynamic changes. Furthermore, it is relevant to consider that the inhibitors used unlikely represent the optimal caspase inhibitor structures for in vivo inhibition of caspase enzymes. Compounds with improved cell penetration properties and/or pharmacokinetic properties may afford greater protection.

The non-selective caspase inhibitor zVAD.fmk, and to a lesser extent, the non-selective caspase inhibitor BodD.fmk and other peptide-based caspase inhibitors, have been used in pharmacologic intervention studies to attenuate tissue/organ injury. The use of these compounds under in vivo and in vitro conditions has been most closely linked to caspase inhibition (Holly et al., 1999; Piot et al., 1999). However, the in vivo selectivity of these compounds for caspase enzymes remains to be determined. Indeed, zVAD.fmk inhibited the cysteine protease enzymes cathepsin B and H (Schotte et al., 1999). Furthermore, putatively selective tetrapeptide inhibitors such as zDEVD.fmk and AcYVAD.fmk also inhibited cathepsin B activity (Schotte et al., 1999). Recognition that the fluoromethylketone (fmk) functional group (contained on both the currently tested compounds of our study), which covalently binds to the active site cysteine residue of caspase enzymes as well as cathepsin cysteine protease enzymes, indicates additional, non-caspase related mechanisms should be considered when regarding the in vivo mechanisms of action of these compounds. While caspase inhibition contributes to the anti-apoptotic effects of these inhibitors, other cysteine proteases or perhaps other enzymes also inhibited by the presence of the fmk functional group, salutary effects may be due to a pleiotropic pharmacologic action rather than specific caspase inhibition. Development of selective, small molecule and reversible non-peptide inhibitors of the different caspase enzymes will contribute to determining the role of caspase activation in myocardial ischemic and reperfusion injury.

In conclusion, our results indicate that pharmacologic treatment of reperfusion-induced cell death by a caspase inhibitor, zVAD.fmk or BocD.fmk, significantly reduces infarct size in vivo in the rat. These results confirm previous observations where caspase inhibitors were administered before the onset of ischemia. Our results also demonstrate for the first time that a caspase inhibitor, zVAD.fmk, administered well after the onset of ischemia, and just prior to reperfusion can also reduce infarct size. Although these results are again positive with respect to infarct size reduction, the extent of salvage is modest; it will be important to assess the efficacy of more selective or cell penetrant caspase inhibitors to determine which caspase(s) is/are principally involved in cardiac apoptosis, and also the extent to which anti-apoptotic compounds may protect the heart form reperfusion-induced injury.

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