Inosine: A Protective Agent in an Organ Culture Model of Myocardial Ischemia

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SUMMARY. Fetal mouse hearts in organ culture provide a model of ischemic-like injury in which the myocardial protective effect of pharmacological agents can be studied independent of blood flow. To investigate the potential protective effect of a diffusible purine under ischemic-like conditions, we used 4 mM inosine in fetal mouse heart organ cultures deprived of oxygen and oxidizable substrates for 1-10 hours. We studied hearts \( n = 258 \) immediately after simulated ischemia (early) and after a 20-hour recovery period (late), by utilizing three indices of myocardial viability. Thallium-201 accumulation is an early marker of myocardial viability during injury, whereas the percentage of lactic dehydrogenase release from hearts to culture medium and the percentage of irreversibly injured myocytes assessed by planimetry of midventricular histological sections are late markers, used after recovery from injury. At 10 hours of injury, thallium-201 accumulation was 38% greater in inosine-supplied hearts, \( 3.50 \pm 0.16 \text{ vs. } 2.54 \pm 0.08 \text{ (counts/min per mg wet weight)/(counts/min per ml medium}) (mean \pm SEM) (P < 0.001). \) After recovery from 10 hours of injury, lactic dehydrogenase release was 29% less in inosine-supplied hearts, \( 35 \pm 3\% \text{ vs. } 49 \pm 3\% \text{ (P < 0.001). } \) After recovery from 8 hours of injury, the percentage of histologically irreversibly injured tissue was 23% less in inosine-supplied hearts, \( 60 \pm 7\% \text{ vs. } 78 \pm 3\% \text{ (P < 0.05). } \) These data indicate that inosine has a protective effect on fetal mouse myocardium during simulated ischemia and suggest that inosine deserves further evaluation. (Circ Res 51:181-188, 1982)

FETAL mouse hearts in organ culture provide a model of ischemic-like insult in which the myocardial protective effect of pharmacological agents can be studied independent of blood flow (Ingwall et al., 1975). Each fetal mouse heart is cultured individually and is maintained at a gas-medium interface on a stainless steel grid (Wildenthal, 1971). Under control conditions (incubation with at least 30% oxygen and with standard culture medium [minimal essential medium (MEM) GIBCO]), the hearts beat spontaneously and respond appropriately to pharmacological agents such as isoproterenol (Chen et al., 1979) or glucagon (Wildenthal et al., 1976). The principal characteristic of this preparation is that the myocardium is nourished by diffusion of nutrients from the culture medium, independent of coronary blood flow.

Myocardial ischemia is simulated with hypoxia and deprivation of oxidizable substrates (glucose, isoleucine, leucine, and valine) from the culture medium (Ingwall et al., 1975). The extent of myocardial necrosis during organ culture is highly reproducible and can be increased by increasing the duration of the ischemic-like insult (Ingwall et al., 1978, 1980; Kloner and Ingwall, 1980). By adding pharmacological agents to the culture medium, the effect of these agents on myocardial protection during simulated ischemia can be studied (Wildenthal et al., 1976). In this model, a protective effect has previously been demonstrated with insulin (Wildenthal et al., 1976), hypothermia (Roeske et al., 1977), graded increases in oxygen (Ingwall et al., 1979), and resupply of oxygen and oxidizable substrates after injury (Kloner and Ingwall, 1980).

In previous studies, the myocardial protective effects of inosine during experimental coronary occlusion have been encouraging. For example, DeVoss and Jones (1979) demonstrated a 25% reduction in canine myocardial infarct size when 2.5 mM inosine was administered 15 minutes after initiation of a 5-hour occlusion of the left anterior descending coronary artery. In these experiments, inosine was also found to increase myocardial perfusion, especially in borderline ischemic myocardium. This increase in myocardial blood flow associated with the administration of inosine was felt to be the primary mechanism for salvaging ischemic myocardium in the canine model. The rationale for our current study was to determine whether an additional myocardial protective effect of inosine during ischemic-like injury can occur independent of coronary flow. Accordingly, we utilized the flow-independent fetal mouse heart organ culture preparation.

In the current study, we investigate the potential myocardial protective effect of inosine, a purine ribonucleoside, by using 4 mM inosine in fetal mouse heart organ cultures deprived of oxygen and oxidizable substrates for 1-10 hours. We studied hearts immediately after simulated ischemia (early) and after a 20-hour recovery period (late) by utilizing three indices of myocardial viability. Thallium-201 (Tl-201)
accumulation is an early marker of myocardial viability during injury (Goldhaber et al., 1981), whereas the percentage of lactic dehydrogenase release from hearts to culture medium (Roeske et al., 1978) and the percentage of irreversibly injured myocytes assessed by planimetry of midventricular histological sections (Kloner and Ingwall, 1980) are late markers used after recovery from injury.

Methods

Sixteen-day pregnant albino mice [strain CR-1 (ICR) BR; Charles River Breeding Laboratories] were killed by cervical dislocation. Hearts weighing approximately 2.5 mg wet weight and measuring 2 mm in diameter were removed, dissected from pericardium and great vessels, and placed on stainless steel grids in Falcon organ culture dishes as described by Wildenthal (1971) and Ingwall et al. (1980, 1982). Approximately 0.5 ml of minimal essential medium (MEM-Grand Island Biological Co.) was placed in the medium well of each culture dish. Control hearts were incubated at 37°C with MEM and 95% O2, 5% CO2. Simulated ischemia was produced by incubating hearts at 37°C in 95% N2, 5% CO2 and MEM which did not contain glucose, isoleucine, leucine, or valine. Immediately after simulated ischemia, the lactate concentration measured on an Automatic Clinical Analyzer (Dupont) was 0.6 mEq/liter using the method of Marbach and Weil (1967); control media contained 0.1 mEq/liter. The pH of the medium measured immediately after simulated ischemia was 7.37 (Corning pH meter 125); the pH of the control medium was 7.38. In hearts treated with inosine, a concentration of 4 mm was used unless otherwise indicated. The experimental groups of fetal mouse hearts are listed in Table 1.

Thallium-201 Accumulation

After overnight equilibration, control and injured hearts, with or without 4 mm inosine, were incubated at 37°C for 1, 2, 3, 4, 5, 6, 8, and 10 hours, with TI-201-labeled medium (3-15 μCi/ml). For each solution, two aliquots each of 2.5, 5, and 10 μl were set aside as standards. At the conclusion of the experiment, medium was removed and the hearts were weighed. Each heart and aliquot of standard solution was counted separately for 1 minute on a gamma well scintillation counter (Searle 1195).

The ratio of TI-201 net accumulation in the hearts relative to the content of TI-201 in the medium was calculated as counts per minute per milligram wet weight of tissue divided by counts per minute per microliter of culture medium (counts/min per mg wet weight)/counts/min per μl medium) (Ingwall et al., 1979; Goldhaber et al., 1981; Pohost et al., 1980). These accumulation ratios were calculated and plotted against time. For each time point and condition, TI-201 accumulation ratios were expressed as the mean ± SEM. The effect of 4 mm inosine was tested on TI-201 accumulation in uninjured oxygenated hearts from 1 to 10 hours, as well as on injured hearts. In one experiment, the effect of varying concentrations of inosine (0.04, 0.4, 4, and 10 mm) on TI-201 accumulation during 10 hours of injury was assessed. In all other experiments, the concentration of inosine was 4 mm.

LDH Loss

Lactic dehydrogenase released from the hearts into the culture medium, a late marker of the extent of irreversible injury, was measured after 20 hours of recovery from 10 hours of injury (Roeske et al., 1978). The 10-hour injury period was chosen because the maximal difference (38%) in thallium accumulation ratios between inosine-supplied medium and no inosine during simulated ischemia occurred at 10 hours. Previous work in our laboratory demonstrated a strong inverse correlation between thallium accumulation and LDH release during simulated ischemia in the fetal mouse heart organ culture preparation (Goldhaber et al., 1981). For each LDH determination, two hearts were homogenized in 0.1 M phosphate buffer, pH 7.4, with 1 mm EDTA and 1 mm β-mercaptoethanol (n = 1). LDH activity was measured in heart homogenates and media in duplicate by the method of Bernstein and Everse (1975). Four combinations of injury/recovery media were assessed: no inosine during injury/no inosine during recovery, no inosine during injury/inosine during recovery, inosine during injury/no inosine during recovery, and inosine during injury/inosine during recovery. LDH loss was expressed as the percentage of LDH released from heart to medium. LDH loss was also determined in control hearts supplied with oxygen and MEM, with or without inosine, after a 30-hour incubation period.

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**TABLE 1**

Summary of Inosine Experiments

<table>
<thead>
<tr>
<th>Marker</th>
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<th>Experimental conditions</th>
<th>Figure</th>
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<td>MEM, O2, inosine</td>
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<tr>
<td>Tl-201</td>
<td>36</td>
<td>MEM, O2, without inosine</td>
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<tr>
<td>Tl-201</td>
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<td>Substrate-depleted MEM, N2, without inosine</td>
<td>2</td>
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<td>Inosine dose-response</td>
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</tr>
<tr>
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<td>LDH</td>
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<td>Inosine during injury/no inosine during recovery</td>
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<td>LDH</td>
<td>9</td>
<td>Inosine during injury/inosine during recovery</td>
<td>4</td>
</tr>
<tr>
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</tr>
<tr>
<td>Histology</td>
<td>8</td>
<td>Inosine during injury/no inosine during recovery</td>
<td>5.6B</td>
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Histopathological Analysis

Inosine-supplied and non-inosine-supplied hearts following 20 hours of recovery from 8 hours of injury were prepared for 1 μm plastic embedded histological sectioning as described in detail previously by Kloner and Ingwall (1980). Following the recovery period, with reoxygenation and repletion of oxidizable substrates, the hearts were placed immediately into Karnovsky’s fixative (2.5% glutaraldehyde and 2.0% paraformaldehyde in cacodylate buffer). The hearts were allowed to fix for 4 hours, rinsed in 0.1 M cacodylate buffer, and then were postfixed in cold 1% osmium for 1 hour. They were rinsed, dehydrated through graded alcohols, and placed in propylene oxide. After this, they were placed in a mixture of propylene oxide and Epon 812, and then Epon 812 alone. The hearts were embedded whole in Epon 812 with the apex pointing toward the tapered end of the capsule. Three 1-μm sections of each heart were obtained midway between apex and base. These sections were cut on a Porter-Blum microtome. Because of the small size of these hearts, each 1-μm section included an entire transverse slice of the ventricles. The sections were then stained with toluidine blue and examined under light microscopy for histological damage. The area of irreversible damage was defined as the area in which cells exhibited pyknotic nuclei, nuclear degeneration, and degeneration of the sarcolemmal membrane. This area was planimetrized by an observer unaware of whether the heart was supplied with inosine. Planimetry was performed by placing a grid in the eyepiece of the microscope and counting the number of boxes overlying the tissue which represented normal vs. necrotic cells.

Statistical Analysis

Data were expressed as mean ± SEM. For the TI-201 experiments done from 1 to 10 hours, a one-way analysis of covariance was used to test the null hypothesis that TI-201 accumulation was equal in inosine-treated hearts. In the dose-response protocol, linear regression by the method of least squares was performed on the TI-201 net accumulation ratio vs. the log of the dose of inosine. For the experiments measuring LDH release, a one-way analysis of variance was used to test the null hypothesis that mean percentage LDH release was equal in all groups. In the histopathology experiment, an unpaired Student’s t-test was used to test the null hypothesis that the percentage of irreversibly damaged cells was equal in inosine-supplied and non-inosine-supplied hearts. Statistical analyses were performed with a commercially available data management package (Dixon and Brown, 1979) and computer system (Digital Equipment Corporation VAX).

Results

Thallium-201 Accumulation

The TI-201 net accumulation ratio was measured for uninjured, oxygenated control hearts treated with (n = 35) and without (n = 38) 4 mM inosine (Fig. 1). Between 1 and 10 hours, myocardial TI-201 content increased over time in both groups, and there was no significant difference with or without inosine in the controls. Since the addition of inosine to the culture medium had no effect on TI-201 accumulation under control conditions, we proceeded to add inosine to the culture medium under conditions of simulated ischemia. In injured hearts treated with (n = 46) and without (n = 51) inosine (Fig. 2), the net TI-201 accumulation ratio during injury peaked at 4 hours and then decreased. The slopes of the curves during injury changed from positive to negative after 4 hours because net TI-201 efflux exceeded net influx. From 1 to 6 hours of ischemic-like injury, there was no difference in TI-201 accumulation in inosine-treated vs. untreated hearts. After 6 hours of injury, more TI-201 remained in the inosine-treated hearts. By 10 hours of injury, TI-201 accumulation was 38% greater in inosine-treated hearts, 3.50 ± 0.16 vs. 2.54 ± 0.08 (counts/min per mg wet weight)/(counts/min per μl medium) (P < 0.001). Although more TI-201 accumulated in insulted, inosine-supplied hearts at 8 and 10 hours, the addition of inosine did not prevent net efflux of TI-201, and, consequently, the slope of the net TI-201 accumulation curve in inosine-supplied injured hearts remained negative from 6 to 10 hours of insult.

After a 10-hour injury, TI-201 accumulation increased linearly in inosine-treated hearts (n = 19) with increasing log concentrations of inosine, compared to hearts not treated with inosine (Fig. 3). This effect was not observed with 0.04 mM inosine but did occur at concentrations of 0.4, 4, and 10 mM inosine. These data fit the equation (TI-201 net accumulation ratio) = 3.53 + 0.65 log mM inosine (r = 0.99).

LDH Loss

After 20 hours of recovery from 10 hours of injury, LDH release from hearts to the culture medium was
FIGURE 2. The TI-201 net accumulation ratio (in counts per min per mg wet weight/counts per min per μl medium) is plotted against time (hours) for two sets of injured hearts: substrate-depleted MEM, N₂, + 4 mM inosine vs. substrate-depleted MEM, N₂ without inosine. The difference between the two sets of data points is significant (P < 0.001).

29% less in inosine-treated hearts (n = 9) compared to controls (n = 9), 35 ± 3% vs. 49% ± 3% (P < 0.001). Additional treatment with inosine during recovery (n = 18) had no significant effect on myocardial salvage.

FIGURE 3. The TI-201 net accumulation ratio (in counts per min per mg wet weight/counts per min per μl medium) is plotted after 10 hours of injury for hearts incubated at 37°C with increasing log concentrations of inosine, ranging from 0.04 to 10 mM. Thallium accumulation increases with increasing doses of inosine. The initial histogram indicates TI-201 accumulation in four hearts not treated with inosine.

FIGURE 4. The percentage of LDH release from hearts to culture medium is measured in four sets of injured hearts: no inosine during injury/no inosine during recovery, no inosine during injury/inosine during recovery, inosine during injury/no inosine during recovery, inosine during injury/inosine during recovery. The hatched areas in the figure indicate that inosine is added to the culture medium. Addition of inosine to the medium during recovery did not significantly increase inosine's protective effect. However, LDH release was 29% less in injured hearts treated with inosine during injury (P < 0.001), compared to hearts not treated with inosine.

(Fig. 4). Thirteen MEM-supplied and oxygenated control hearts were incubated with or without inosine for 30 hours and had typical control LDH release values of 5 ± 1%.

Histopathological Analysis

After 20 hours of recovery from 8 hours of injury, the percentage of irreversibly damaged cells was 23% less in inosine-treated hearts (n = 8) compared to controls (n = 8), 60 ± 7% vs. 78 ± 3% (P < 0.05) (Figs. 5 and 6). Figure 6A demonstrates a midventricular histological section of an injured heart in which inosine was not added to the culture medium. Almost all of the cells are necrotic except for a thin rim of viable tissue (darkly stained cells). The planimetered area of irreversibly damaged cells was 79%. Figure 6B demonstrates a midventricular histological section of an injured heart in which inosine was added to the culture medium during injury. The rim of viable epicardium is much larger in this section, compared to the heart in Figure 6A. Furthermore, there are...
patchy areas of viable cells throughout the section in which inosine was added to the culture medium. The planimetric area of irreversibly damaged cells was 57%.

Discussion

The present study uses three markers to show that supplying inosine during simulated ischemia (before recovery) has a protective effect in cultured fetal mouse hearts. The addition of inosine only during recovery from ischemic-like injury did not appear to salvage additional myocardium. It should be noted that this preparation derives its nutrient supply from the culture medium and utilizes deprivation of oxygen and oxidizable substrates rather than true ischemia to produce injury. Unlike ischemia in intact hearts, exogenous neural or humoral factors are not present. While having the disadvantage of incompletely modeling ischemia, this preparation allows the study of the effects of injury on tracer kinetics in myocardium independent of blood flow (Pohost et al., 1980). The percentage of decreased Tl-201 accumulation after injury, before recovery, has been shown in noninosine-injured hearts to correlate closely with the percentage of LDH release measured after recovery from injury (Goldhaber et al., 1981). Measurement of Tl-201 uptake, therefore, allows early evaluation of the viable cell mass immediately after injury, before a recovery period. LDH release, a late marker of myocardial injury, does not occur during the 10-hour injury period. However, after 20 hours of recovery with reoxygenation and repletion of oxidizable substrates, LDH loss from myocytes to medium is essentially complete.

The use of thallium accumulation as a marker of cell viability at multiple time points provided a prospective indicator of inosine’s potentially beneficial effects, before a recovery period. In Figure 2, it is apparent that a significant protective effect does not occur until 8 hours of insult. Previous studies from our laboratory have shown that this 8 hour period of insult results in approximately 40% LDH loss from hearts to culture medium (Goldhaber et al., 1981). In the current investigation, a beneficial effect of inosine could not be shown at lesser degrees of insult. This finding is consistent with our previous experience using the fetal mouse heart organ culture preparation. In this model, we have found that it is difficult to demonstrate myocardial salvage with any pharmacological agent unless the initial injury is severe.

Previous studies have reported that the concentration of endogenous inosine increases in the ischemic heart. After electrically induced ventricular fibrillation (Parker et al., 1976), the concentration of endogenous inosine in canine left ventricle increases from nondetectable to greater than 2.0 μmol/g wet weight at 35 minutes after cardiac arrest. The increase in inosine concentration was more than four times greater than the increase in inosine monophosphate, hypoxanthine, or adenosine. After canine coronary artery occlusion, endogenous inosine increased from nondetectable to more than 0.5 μmol/g wet weight at 30 minutes (Jones et al., 1976). Increases in the concentration of inosine monophosphate, hypoxanthine, and adenosine were much smaller than the increase in endogenous inosine after the onset of ischemia.

Previous studies have also shown that inosine has potent inotropic effects. Buckley et al. (1959) used an isolated canine left ventricle preparation to demonstrate that exogenous inosine exerts a positive inotropic effect after the onset of acute left ventricular failure. The inotropic effect of inosine occurred independent of heart rate, chamber size, or coronary supply. More recently (Jones et al., 1977), it has been shown in the in situ canine heart that exogenous inosine in concentrations of 10 mm or greater, exerts a positive inotropic effect that is not mediated through adrenergic mechanisms. In another series of canine coronary artery occlusion experiments (Thomas et al., 1979) it was demonstrated that inosine infusion increases myocardial contractile force by 32% in the ischemic zone, by 41% in the borderline ischemic zone, and by 42% in a nonischemic zone. These increases in myocardial contractile force occurred without any significant changes in the concentration of adenosine triphosphate or creatine phosphate. Using a model of porcine coronary artery occlusion, Woollard et al. (1981) found that administration of
Figure 6. A histological section of non-inosine-supplied fetal mouse myocardium (Fig. 6A) is compared with inosine-supplied tissue (Fig. 6B) after recovery from simulated ischemia (see text).

Inosine caused peripheral vasodilation with hypotension, coronary vasodilation and a positive inotropic effect. Regional myocardial performance was increased more in the ischemic myocardium than in the normally perfused myocardium.

As mentioned in the introduction, exogenous inosine has been shown to increase myocardial blood flow and to salvage 25% of ischemic myocardium during canine coronary artery occlusion (Devous et al., 1979). This quantitation of myocardial salvage was determined by staining with nitro blue tetrazolium. In our histological studies of fetal mouse myocardium, we observed an essentially identical 23% salvage of injured myocardial, despite the obvious differences in species, maturity of tissue, and method of supplying inosine. Previous studies of inosine have emphasized increased coronary blood flow as an important mechanism to explain inosine's beneficial effects. These studies, by nature of the experimental models utilized, have been unable to separate the direct cellular effects of inosine from inosine's effects on coronary blood flow. Thus, our study extends the previous work in this field to a model which is independent of coronary flow. We have shown that in considering the mechanisms whereby inosine salvages myocardium, there is a direct protective component which is not dependent on coronary flow and which accounts for 23% to 38% myocardial salvage. These observations should be useful in defining mechanisms by which exogenous inosine protects the ischemic myocardium.
As a therapeutic agent, inosine has been used most extensively to protect kidneys during warm ischemia. Experimental work established inosine as beneficial in preservation of renal function in rats (Fernando et al., 1976, 1977a, 1979) and dogs (Fernando et al., 1977b; Kaufman and Woo, 1978) undergoing warm renal ischemia. However, several groups have found no additional renal protection with inosine (Marshall et al., 1978; Casali et al., 1979). Nevertheless, the results of preliminary, uncontrolled, unblinded clinical studies in which inosine was used to preserve renal function during ischemic renal surgery have been encouraging (Wickham et al., 1978a, 1978b, 1979). No clinical toxicity from inosine has been reported.

The finding of myocardial salvage in the injured fetal mouse heart treated with inosine during injury indicates that this purine nucleoside has a protective effect. The current investigation was limited to documentation of inosine’s effects during ischemic-like injury and after recovery from injury. Inosine’s mechanism of action was not studied, nor were other purines tested for potential myocardial salvage. Whatever inosine’s mechanism of action might be, it was not dependent on flow in this organ culture preparation. We present our findings at this time to stimulate further research with purines as potentially clinically applicable myocardial protective agents.

We wish to thank Thomas W. Smith, M.D., for reviewing this manuscript and Nancy Carlson for excellent technical assistance. We thank Robert Lopez, Chief of the Chemistry Laboratory, Brigham and Women’s Hospital, for the assays of lactate concentration in the culture media that he performed. The thallium was kindly supplied by New England Nuclear Corporation.

These studies were supported by U.S. Public Health Service Grants HL21751, HL26215, and HL23140. Dr. Goldhaber was supported by NRSA Training Grant HL07049. Drs. Pohost, Kloner, and Ingwall are Established Investigators of the American Heart Association.

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Received February 22, 1982; accepted for publication May 21, 1982.

References

Goldhaber et al. / Protective Effect of Inosine

187

INDEX TERMS: Inosine • Ischemic-like insult • Fetal mouse hearts • Thallium • Lactic dehydrogenase • Histology
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*Circ Res.* 1982;51:181-188
doi: 10.1161/01.RES.51.2.181

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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