Lack of Effect of Methylprednisolone on Lysosomal and Microsomal Enzymes after Two Hours of Well-Defined Canine Myocardial Ischemia

FRED F. KENNETT AND WILLIAM B. WEGLICKI

SUMMARY Myocardial ischemia was produced for 2 hours by coronary ligation in 11 dogs pretreated with methylprednisolone (MP, 30 mg/kg). Myocardial blood flow (MBF) was measured with microspheres (15 µm) in each tissue sample used for enzymatic analysis. Homogenates of these tissue samples were separated by ultracentrifugation into lysosome-rich and microsomal fractions and were analyzed for N-acetyl-β-glucosaminidase (NAGA), β-glucuronidase (β-gluc), rotenone-insensitive-NADH-cytochrome c reductase (RINCR), and cytochrome oxidase. The enzymatic data from centrifugal fractions were grouped according to MBF values for statistical analysis of inter-group effects of ischemia. Significant losses (P < 0.001) of NAGA and β-gluc were seen in all MP-treated lysosome-rich particulate fractions that were isolated from zones demonstrating MBF values less than 25% of control (L-ischemia). Similar significant losses (P < 0.001) of RINCR were seen in microsomal fractions from L-ischemia zones. Samples with MBF values greater than 25% but less than 75% of control (M-ischemia) also demonstrated significant decreases of lysosomal and microsomal enzymatic activity in specific fractions. When the data of the above MP-treated group were compared with the untreated control group, no significant intergroup effects of treatment with MP were observed. In addition, enzymatic data (NAGA, RINCR) were normalized prior to performing linear regression analyses; percent loss of particulate enzymatic activity was plotted against percent decrease in MBF. The effects of 2 hours of ischemia on the above biochemical parameters were comparable between untreated and MP-treated groups. Finally, when myocardial samples were grouped according to similar levels of MBF, statistical analysis using the general linear models procedure revealed no beneficial effect of MP treatment on changes in lysosomal hydrolases, microsomal RINCR, or latency of lysosomes.

TREATMENT with pharmacological doses of anti-inflammatory corticosteroids has been reported to reduce the size of myocardial infarction in experimental animals. Recent reports have indicated beneficial effects of treatment with corticosteroids on various parameters commonly used to assess ischemic tissue damage: decrease in S-T segment elevation of the electrocardiogram,1-3 retention of myocardial creatine phosphokinase,1-4 histological estimates of injury,1,4,5 favorable cardiovascular hemodynamics and blood flow,6-10 and myocardial lysosomal2,3,5,7 and plasmalemmal11 stabilization. However, other studies have suggested no beneficial effects of treatment with methylprednisolone (MP) using similar hemodynamic12-15 or biochemical12-16 parameters. The controversy that has arisen about the possible beneficial effect of methylprednisolone on lysosomal parameters is understandable because differing biochemical methodologies have been employed by various investigators.2,3,5,7,14 We have endeavored to improve the isolation of lysosomes and microsomes16 from myocardial tissue and have studied changes in the enzymes of these organelles during well-defined ischemia7,17-19 to provide a better baseline for assessing effects of pharmacological interventions. The present study reports the effects of prior administration of MP on these enzymatic activities in experimental animals after acute occlusion of the left anterior descending coronary artery for 2 hours, using the methodology described in the preceding article.19

Methods

Surgical Procedure

Briefly, in 11 MP-treated dogs that had negative blood tests for heartworms, anesthesia was induced by intravenous injection of sodium pentobarbital, 27 mg/kg (Diabutal, Diamond Lab., Inc.). Intubation was achieved with auffed endotracheal tube and respiration was supported with a positive pressure respirator (Harvard Apparatus Co., Inc.). The femoral artery and vein were isolated for insertion of arterial and venous catheters; arterial pressures were monitored by a Beckman R411 Dynograph recorder. Needle electrodes were placed subcutaneously for continuous monitoring of the electrocardiogram (ECG) and changes in lead II were recorded on this instrument. A left thoracotomy...
was performed for exposure of the heart and the left anterior descending coronary artery was dissected free of connective tissue immediately distal to the first diagonal branch. The left atrial appendage was incised and a catheter was placed into the left atrium for injection of microspheres. At the appropriate time, a nylon ligature was placed around the LAD; occlusion of the LAD was produced with this ligature.

**Administration of Methylprednisolone (MP)**

Thirty minutes prior to ligation of the LAD, methylprednisolone (Solu-Medrol, Upjohn) was administered (30 mg/kg) over a 1-minute period via the femoral vein under constant hemodynamic monitoring.

After 2 hours of ischemia, each heart was excised and placed in cold buffer (0.25 M sucrose, 10 mM imidazole at 4°C). Tissue samples from control and ischemic endocardium were disrupted and fractionated by centrifugation as described in the preceding article.

**Analysis of Myocardial Blood Flow (MBF)**

Radioactive microspheres (15 ± 5 μ in diameter, Nuclear Products) were used to measure regional MBF. Each injection contained approximately 1.76 x 10^6 microspheres that were diluted in 55% wt/vol sucrose to a final volume of 8 ml. The technique of administration was described in the previous article. All samples were counted in a Beckman 300 Gamma Counter.

These samples were grouped according to their percent of control value. Samples having greater than 75% of control MBF were designated highest control (H-control); samples having greater than 25% and less than 75% of control blood flow were classified as moderately ischemic (M-ischemia); samples with less than 25% of control MBF were considered severely ischemic and were placed in the lowest flow group (L-ischemia).

**Enzymatic Assays**

Assays of N-acetyl-β-glucosaminidase (NAGA) and β-galactosidase (β-gal), cytochrome oxidase, rotenone-insensitive NADH-cytochrome c reductase (RINCR), and latency of lysosomes and protein were performed on homogenates and all centrifugal fractions.

**Statistical Analysis of Data: Three MBF Groups**

Means and standard errors were determined for each biochemical parameter in each fraction. The changes in enzymatic activities or protein values between tissue samples were tested for significance at different levels of ischemia (between H-control and M-ischemia, and between H-control and L-ischemia) by analysis of variance. In addition, significant differences were sought between untreated and MP-treated groups (e.g., between H-control samples of both experimental groups). All recovered enzymatic activities and proteins of the three MBF groups (H-control, M-ischemia, and L-ischemia) were averaged to give the total activities of these parameters. Means were tabulated for each fraction of both untreated and MP-treated groups to test for possible drug effects in the individual fractions (pellets and supernatant) of the MP-treated group at the three levels of MBF. These analyses were performed on an IBM 370-145 computer using the Statistical Analysis Systems package. The level of significance within (control vs. ischemia) each group or between (treatment differences) groups was determined by Student's t-statistic using pooled estimates of the standard deviations.

**Statistical Analysis of Data: Linear Correlations**

Data for MBFs from each heart were plotted as percents of the highest of two control flow values (100%) of untreated and MP-treated groups. Also, all enzymatic values of the endocardial samples exhibiting the highest MBF (H-control from untreated and MP-treated groups) were considered as the 100% baseline. Data points and regression lines were plotted by the Tektronix 4006-1 Graphics Terminal of the IBM 370 computer. The abscissa represents the percent of control MBF and the ordinate shows the percent of control enzymatic activity. The ordered pair (100%, 100%) is excluded for each of these parameters. Means were tabulated for each of these parameters. Means were tabulated for each of these parameters. Means were tabulated for each of these parameters. Means were tabulated for each of these parameters. Means were tabulated for each of these parameters.

**Hemodynamic Parameters**

The heart rates (beats/min ± SEM) during the pre-thoracotomy period were 130 ± 7 for untreated and 124 ± 7 for MP-treated groups; these differences were not significant. In the post-thoracotomy period (prior to occlusion) the heart rates for the untreated group averaged 148 ± 6 and for the MP-treated group, 144 ± 7; thus there was an increase in heart rate of approximately 20 beats/min for both untreated and MP-treated dogs after the stress of the surgical procedure; no significant differences were noted at this time. During the post-occlusion period, no significant differences in heart rate were detected between experimental groups (untreated: 145 ± 8 at 5 minutes to 138 ± 6
at 120 minutes; MP-treated: 139 ± 7 at 5 minutes to 130 ± 5 at 120 minutes).

The intravenous administration of MP as a bolus caused a transient but significant decrease in the arterial pressure (AP) for all dogs; the fall in AP (129 ± 5 mm Hg to 110 ± 8 mm Hg) lasted approximately 5 minutes before returning to control values. This observation confirmed results reported by Masters et al.,* who used MP (40 mg/kg). However, no significant differences were noted in the degree of depression of AP between the untreated and MP-treated groups in the pre-occlusion (untreated: 119 ± 5; MP-treated: 121 ± 3) or the post-occlusion (untreated: 112 ± 3 at 5 minutes to 104 ± 5 at 120 minutes; MP-treated: 108 ± 4 at 5 minutes to 103 ± 6 at 120 minutes) periods. Thus, the administration of MP did not affect significantly these hemodynamic indices between groups under these experimental conditions.

Analysis of Myocardial Blood Flow (MBF)

Table 1 lists the MBFs of MP-treated dogs. On gross inspection, the MP-treated group exhibited visible ischemic zones that were similar in appearance to those of the untreated group. Also, one dog of the MP-treated group showed no apparent zone of infarction after 2 hours of acute occlusion of the LAD. The values in Table 1 are presented as absolute flow (ml/min per g) and as the percentage of the highest control value from MP-treated hearts. The MP-treated MBFs represent 19 H-control, 8 M-ischemia, and 13 L-ischemia samples. In the M-ischemia zones, the MBFs of MP-treated dogs fell to approximately 50% of control MBF; in the L-ischemia zones the MBFs were less than 10% of control. Thus, the MBFs of H-control, M-, and L-ischemia zones of untreated and MP-treated groups were comparable, and no statistically significant differences could be found between both groups.

Hematological Parameters

The hematocrits (untreated: 34.5 ± 6; MP-treated: 35.1 ± 8) and white blood cell (WBC) counts of untreated and MP-treated groups showed no statistically significant differences; the untreated dogs showed an average decrease in the peripheral WBC counts over the 2-hour experiment (12,600 to 10,300), whereas the MP-treated dogs showed unchanging WBC counts (10,900 to 10,900).

Comparison of Changes in Proteins

Table 2 lists the sum of total protein that was recovered from all centrifugal fractions of the MP-treated group. Total decreases in protein per gram of wet weight were highly significant (P < 0.001, represented by asterisks) in the M-ischemia and the L-ischemia zones when compared with the H-control area.

No significant differences were found in the 1,000 g pellets. Differences for post-1,000 g fractions were greatest in the L-ischemia zones, where highly significant decreases in content of protein of all post-1,000 g particulate (P < 0.001) and supernatant (P < 0.001) fractions were found. The proteins of the M-ischemia zone showed significant decreases in all post-1,000 g fractions. Between untreated and MP-treated groups, the only significant difference (represented by a cross) occurred in the 20,000 g fractions of the H-control zones (P < 0.05); all other values for total protein in each fraction of both experimental groups were not statistically different from each other.

Comparison of Lysosomal Enzymatic Changes

The highest specific activities and percent distribution of total lysosomal activities were found in the 9,000 g and 20,000 g pellets of H-control tissue; in addition, the post-1,000 g pellets contained more than half of the total activity of lysosomal enzymes; the 1,000 g pellets usually contained 25–30% of total activity of lysosomal enzymes, while the supernatant fractions (H-control) contained 13–16% of total lysosomal enzymatic activity.

NAGA

In Table 3A, data are presented for the changes in sum total activity of NAGA and activity in all particulate and supernatant fractions to permit accounting of redistribution (e.g., into the 1,000 g pellet and 140,000 g supernatant) and loss (from total and post-1,000 g particulate fractions) of all lysosomal activity.

Highly significant sum total losses of activity of NAGA (P < 0.001) from L-ischemic zones are seen; the M-ischemic areas had similar sum total losses

| Table 1 Analysis of Myocardial Blood Flow during Ischemia of MP-Treated Dogs |
|-----------------------------|------------------|------|
| Group          | ml/min per g | % control |
| H-control       | 1.39 ± 0.15    | 90.0 ± 2.1 |
| M-ischemia      | 0.86 ± 0.15    | 50.0 ± 4.5 |
| L-ischemia      | 0.15 ± 0.04    | 9.6 ± 2.0  |

<p>| Table 2 Total Protein in Myocardial Fractions from MP-Treated Dogs during Ischemia |
|-----------------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Fraction</th>
<th>H-control</th>
<th>M-ischemia</th>
<th>L-ischemia</th>
</tr>
</thead>
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<tr>
<td>1,000 g</td>
<td>43.2</td>
<td>40.7</td>
<td>44.4</td>
</tr>
<tr>
<td>2,500 g</td>
<td>16.0</td>
<td>13.8</td>
<td>11.3</td>
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<td>9,000 g</td>
<td>8.3</td>
<td>6.8</td>
<td>5.8</td>
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<tr>
<td>20,000 g</td>
<td>4.2†</td>
<td>3.5</td>
<td>3.1</td>
</tr>
<tr>
<td>140,000 g</td>
<td>9.8</td>
<td>7.8</td>
<td>6.2</td>
</tr>
<tr>
<td>Supernatant</td>
<td>83.7</td>
<td>72.8</td>
<td>68.3</td>
</tr>
<tr>
<td>Total</td>
<td>165.0</td>
<td>145.3</td>
<td>139.2</td>
</tr>
</tbody>
</table>

* P < 0.05, ** P < 0.001, † (P < 0.05) intergroup difference.
The total activity of NAGA in the 1,000 g pellet of the L-ischemia area was increased significantly \((P < 0.001)\) compared to the H-control area, whereas the 1,000 g pellet of the M-ischemia zone showed a slightly significant loss of NAGA \((P < 0.05)\) when compared to the H-control zone.

In the L-ischemia post-1,000 g particulate fractions, highly significant losses of NAGA \((P < 0.001)\) were noted. Similar changes in significance were seen in all M-ischemia fractions. The L-ischemia supernatant fraction showed a dramatic increase \((P < 0.001)\). The supernatant fraction of the M-ischemia area showed no significant change, suggesting that either redistribution into the supernatant fraction did not occur or that washout of NAGA was more efficient in this zone with higher mean MBFs.

No significant effect could be ascribed to MP treatment when these ischemic changes in NAGA between all fractions of untreated and MP-treated groups were tested statistically.

\(\beta\text{-gluc}\)

Table 3B lists the sum total activity of \(\beta\text{-gluc}\) for all fractions. Highly significant total loss of \(\beta\text{-gluc} \quad (P < 0.001)\) from the L-ischemia zone is present; no significant total loss of \(\beta\text{-gluc}\) was seen in the M-ischemia zone of the MP-treated group. No effect of MP treatment on these changes in total \(\beta\text{-gluc}\) could be detected between untreated and MP-treated groups.

No significant changes were seen in the 1,000 g pellets. On the other hand, highly significant losses of \(\beta\text{-gluc} \quad (P < 0.001)\) were seen for all post-1,000 g pellets in the L-ischemia area. The supernatant fraction showed highly significant increases of \(\beta\text{-gluc} \quad (P < 0.001)\) in the L-ischemia zone; only a slightly significant increase of \(\beta\text{-gluc} \quad (P < 0.05)\) was found for the M-ischemia zone.

Nevertheless, when these changes in \(\beta\text{-gluc}\) for all particulate and supernatant fractions of untreated and MP-treated groups were analyzed statistically, no significant effect of MP treatment could be found.

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Nevertheless, when these changes in \(\beta\text{-gluc}\) for all particulate and supernatant fractions of untreated and MP-treated groups were analyzed statistically, no significant effect of MP treatment could be found.

**Comparison of Microsomal Enzymatic Changes: RINCR**

Table 4A shows the changes of RINCR during ischemia. The highest specific activities of this enzyme were found in the 140,000 g pellets and no activity was found in the supernatant fractions due to the membrane-bound nature of this enzyme; also, more than 40% of the total activity of this enzyme was found in the 140,000 g "microsomal" fraction.

As was seen previously for the lysosomal enzymes (Table 3), a significant sum total loss of RINCR \((P < 0.001)\) was seen in the L-ischemia area. The sum total loss of RINCR in the M-ischemia zone was less significant \((P < 0.05)\). No statistically significant effect of MP treatment on these changes in sum total RINCR could be found between untreated and MP-treated groups.

No differences were seen in the 1,000 g pellets of M-ischemia and L-ischemia zones. The post-1,000 g pellets of the L-ischemia area showed highly significant losses of RINCR \((P < 0.001)\). Statistical analyses failed to demonstrate any significant MP-treatment differences between all post-1,000 g fractions of both untreated and MP-treated groups.

**Comparison of Mitochondrial Enzymatic Changes: Cytochrome Oxidase**

Table 3B lists the total activities of cytochrome oxidase in all fractions. Activities are reported only for the pellets, since negligible activities were found in the 140,000 g supernatant fractions. The H-control zone had 95% of total cytochrome oxidase activity in the lower speed fractions \((1,000 g \text{ to } 9,000 g)\).

The sum total losses of activity of cytochrome oxidase were significant in M-ischemia and L-ischemia areas.

The 1,000 g and 2,500 g fractions of the L-ischemic area showed significant decreases in cytochrome oxidase. The M-ischemic area demonstrated a significant decrease \((P < 0.05)\) in the 2,500 g fraction.
characteristics as those not receiving the drug; a decrease in the activity of cytochrome oxidase in the mitochondria-enriched fractions and a decrease in sum total activity per gram of wet weight from ischemic tissue. No significant effect of MP treatment was noted between groups for these ischemia-induced changes in activity of cytochrome oxidase.

In summary, the enzymes of the lysosomes, microsomes, and mitochondria demonstrated: (1) diminished particulate activity in the various ischemic post-1,000 g fractions, (2) decreases in total activity per gram of wet weight in the ischemic zones, (3) characteristic shifts of activity (NAGA and β-gluc) from the particulate fractions to the supernatant fractions in L-ischemic zones, and (4) no evidence for any significant beneficial effect of MP treatment on the enzymes and proteins from ischemic endocardium when grouped according to similar levels of MBF.

Linear Statistical Models for Comparison of Enzymatic Changes during Ischemia

The above Tables (3 and 4) represent the values for enzymatic data (total activity/g) when the samples were grouped into H-control (100% to 75% MBF), M-ischemia (75% to 25% MBF), and L-ischemia zones (less than 25% MBF). Thus the data were arranged as 2 × 3 factorials in blocks for statistical analyses of intragroup and treatment differences. To relate the extent of these enzymatic changes (as percent of H-control enzymatic activity) to the entire spectrum of diminished MBF, the data were normalized as described in Methods; each point of enzymatic data (n = 30 for the untreated group and n = 30 for the MP-treated group) was plotted against the MBF in the endocardial sample from which it was obtained and data were analyzed using linear statistical models. This treatment of the enzymatic data corrects for some of the biological variability in levels of activity from animal to animal, and allows for the abolition of the constraints placed on the data by the grouping procedure.

Figure 1 demonstrates the linear regression analysis of changes in NAGA in lysosome-enriched particulate and supernatant fractions. Figure 1A plots the percent of total NAGA (ordinate) against the percent of control MBF (abscissa) for the 9,000 g fraction of untreated (solid line) and MP-treated (dashed line) groups. Both lines were highly significant (P < 0.001) and demonstrated high linear correlation coefficients; r = 0.88 for untreated and r = 0.89 for MP-treated groups; the y-intercepts at 0% MBF were 59.7% for untreated and 54.5% for MP-treated groups. Figure 1B shows the regression lines of the 20,000 g fractions from the two experimental groups; both lines were highly significant (P < 0.001) with coefficients of r = 0.91 for the untreated and r = 0.89 for the MP-treated groups; the y-intercepts at 0% MBF were 53.0% for untreated and 50.1% for the MP-treated groups. Figure 1C demonstrates the regression lines for the 140,000 g pellets; for both untreated and MP-treated groups, the lines were highly significant (P < 0.001), with coefficients of r = 0.88 and r = 0.89, respectively; the y-intercepts at 0% MBF were 49.5% and 46.7%, respectively. Thus, all particulate fractions that are presented here as linear models showed a loss of total activity of NAGA that correlated significantly with decreasing MBF. Figure 1D represents the increase in total activity of NAGA in the supernatant fractions of both experimental groups expressed as a percent of H-control; the untreated and MP-treated groups yielded significant regression lines, P < 0.004 and P < 0.008, respectively, and the correlation coefficients of the supernatant fractions were r = 0.53 for untreated and r = 0.64 for the MP-treated groups; the y-intercepts at 0% MBF occurred at 145% of control for untreated and 169% for MP-treated groups. Using linear models, with statistical testing for significance (Methods), pretreatment with MP did not affect significantly these characteristic losses of NAGA activity from lysosome-rich fractions or the increases of activity in the supernatant fractions.

Figure 2 demonstrates the regression analysis of changes in percent of total RINCR plotted against changes in percent of control MBF. Figure 2A represents the changes in the percent of total RINCR activity in the 20,000 g fractions relative to decreasing MBF in untreated and MP-treated groups; the activity of RINCR decreases as the severity of ischemia increases. The regression lines were significant (P < 0.001) for both groups; correlation coefficients were r = 0.84 for untreated and r = 0.89 for MP-treated groups; the y-intercepts at 0% MBF were 56.9% for untreated and 48.4% for MP-treated groups. Statistical analysis of these regression lines demonstrated a significant negative effect (P < 0.05) of MP-treatment, i.e., a greater loss of RINCR in the MP-treated group. This result is in contrast to the lack of significant intergroup differences described in Table 4 for this fraction. Figure 2B plots the loss of RINCR against MBF for the 140,000 g fractions of both groups. It should be noted that the activity of RINCR was concentrated in this mitochondrial fraction; the changes in activity that were seen at relatively high MBFs (25% to 75% MBF) suggest that this enzyme was particularly sensitive to the effects of ischemia. Statistical analysis of both lines revealed significance (P < 0.001), and correlations were r = 0.81 for untreated and r = 0.93 for MP-treated groups; the y-intercepts at 0% MBF were 45.5% for untreated and 40.2% for MP-treated dogs. Statistical analyses showed no differences between groups, indicating that MP treatment could not prevent the characteristic losses of activity of RINCR during ischemia.

Figure 3 depicts the relationship of the percent free activity of NAGA with decreasing MBF. It
should be noted that these values are not plotted as a percent of control. Percent free activity is an expression of latent enzymatic activity of the lysosome and the lower the value for percent free activity, the more intact is the lysosome. The loss of free activity from the 2,500 (3A), 9,000 (3B), and 20,000 (3C) g fractions appeared linear with respect to decreasing MBF; this was confirmed by linear regression analysis. Statistical analyses between groups showed that none of these regression lines (Fig. 3, A–C) were significantly different for this parameter. However, the data for the 140,000 g pellet (3D) did not appear as linear as in the L-ischemia zone (Fig. 3, A–C) (< 25% MBF); analysis of residuals revealed a failure of the predicted line to fit the observed data. Spline regression analysis was performed on H-control, M-ischemia, and L-ischemia zones of untreated and MP-treated dogs. This analysis showed that the lines that were plotted for the H-control (>75% MBF) and M-ischemic (25% to 75% MBF) zones were not significantly different from each other; however, the line of the 140,000 g pellets of the L-ischemic zones (<25% MBF) were different (P < 0.05) from the H-control.
and M-ischemia lines; this confirmed that the loss of lysosomal latency of this fraction occurred only under conditions of severe ischemia, unlike the changes in the other (Fig. 3, A–C) particulate fractions. This finding suggests a threshold phenomenon for latency of the 140,000 g pellets (untreated and MP-treated) below 25% of control MBF.

Discussion

In recent years, various drugs have been used to attempt to influence biochemical parameters characteristic of myocardial ischemia.23 The application of anti-inflammatory steroids to this problem was stimulated by reports of their stabilization of a wide variety of biological and synthetic membrane systems.24

Anti-inflammatory steroids have been reported to reduce the severity and extent of myocardial ischemic damage secondary to the ligation of a coronary artery in experimental animals.23 Several investigators have reported stabilization of myocardial lysosomes by treatment with anti-inflammatory steroids during ischemia;2,3,5,7 this work emphasized favorable alterations in redistribution of lysosomal enzymes (from particulate to soluble fractions); histochemical evidence was presented to show that acid hydrolases were less altered by ischemia after treatment.5

Other investigators have not been able to find biochemical evidence of a significant beneficial effect of treatment with anti-inflammatory steroids during hypoxia10 or ischemia12–14 of the myocardium. In one study, the interesting point was made that methylprednisolone, by a stabilizing effect on myocardial membranes, may have only delayed the release of myocardial enzymes from the injured heart.15 It is of interest that studies of the localization of labeled glucocorticoid in normal and ischemic myocardial tissue of the cat demonstrated recovery of the largest proportion of radioisotopic label in a fraction enriched with myocardial sarcolemma.11 Another group collected cardiac lymph during a 2-hour period of ischemia and reported that methylprednisolone did not reduce but may have augmented release of acid phosphatase.14

Our choice of marker enzymes for lysosomal activity included N-acetyl-β-glucosaminidase, since recent experimental work has established this hy-
drolase to be an excellent marker of myocytic lysosomes. Membrane-bound enzymes (mitochondrial and microsomal) are useful for quantifying cross-contamination of lysosome-enriched fractions, in addition to providing further parameters for assessing injury of nonlysosomal membranes. We investigated changes in activity of these enzymes in particulate and supernatant fractions in order to rule out a redistribution effect. It is important to note that other investigators frequently discard the nuclear pellet or other low speed fractions without reporting enzymatic activity or proteins. To illustrate this problem, in Table 3A the activity of NAGA was significantly elevated in the 1,000 g pellet of the L-ischemic zone. Had this pellet been discarded, the sum total loss of NAGA in this zone would have appeared even greater than the loss which we reported.

In Table 2, the changes in total protein per gram of wet weight are listed. The changes in sum total
protein per gram of wet weight of the L-ischemia zone show highly significant decreases in post-1,000 g fractions; the largest decrease was seen in the supernatant fraction. We could not find any significant redistribution of proteins into the 1,000 g pellet to explain these losses. The possible reasons for these changes include early edema,26,27 washout of intact proteins,28 or proteolysis.29 Nevertheless, when these data for changes in protein per gram of wet weight were examined statistically for an MP treatment effect, no significant differences were found between either the L-ischemia or M-ischemia zones of both untreated29 and MP-treated groups.

Table 3 lists the levels of significance for losses of NAGA and β-gluc from M- and L-ischemia zones. The L-ischemia areas, with a mean of 10% of control MBF over the 2-hour period of ischemia, always displayed the highest levels of significance for losses of sum total \((P < 0.001)\) and post-1,000 g particulate \((P < 0.001)\) activity of both NAGA and β-gluc; both untreated29 and MP-treated dogs showed similar degrees of loss of lysosomal enzymes, and statistically significant differences for MP treatment could not be detected in the L-ischemia zones. Very similar sum total and particulate losses of NAGA were seen in the M-ischemia zones of both groups. β-gluc has been shown to be a less specific marker of lysosomes of the myocyte than NAGA;26 the sum total losses of β-gluc were less significant and of lower magnitude \((299.0 \text{ to } 276.2, P < 0.05, \text{ untreated}; 285.0 \text{ to } 280.0, P = \text{NS, MP treated})\) than the sum total losses of NAGA \((723.2 \text{ to } 651.3, P < 0.001, \text{ untreated}; 671.0 \text{ to } 577.7, P < 0.01, \text{ MP treated})\). Nevertheless, there was no significant effect of MP treatment on sum total losses, particulate losses, or supernatant increases for β-gluc when grouped according to H-control, M-ischemia, and L-ischemia zones.

The sum total losses and particulate losses of RINCR in the L-ischemia zones of both groups were not significantly different, indicating no effect of MP treatment. Even though losses of RINCR from fractions of the M-ischemia zone showed higher levels of significance in the untreated group than in the MP-treated group, there was no statistically significant MP treatment effect. Similar data were obtained for cytochrome oxidase which was analyzed in fewer animals than RINCR. Thus, the two membrane-bound, non-lysosomal enzymes (RINCR and cytochrome oxidase) reflected progressive losses in moderately and severely ischemic zones; this provides evidence for lack of an MP-treatment effect on microsomal and mitochondrial membrane-bound enzymes during ischemia.

Our decision to plot the changes for two enzymes (NAGA and RINCR) against alterations in MBF was prompted by the concern that the above grouping into H-control, M-ischemia, and L-ischemia zones might obscure subtle changes characteristic of ischemia, especially in the M-ischemia zones. Using linear regression analyses with changes of MBF plotted on the abscissa (Figs. 1–3), the entire spectrum of normal and ischemic conditions could be related to progressive alterations in enzymes and latency; in our opinion this method of considering the data provides a sensitive and powerful statistical assessment of the extent of untreated and MP-treated changes in enzymes during ischemia. In Figures 1 and 2 we show that the regression lines for losses of NAGA and RINCR from lysosome- and microsome-rich particulate fractions were not significantly different; also, the ischemia-induced solubilization of NAGA into the 140,000 g supernatant fractions was not significantly different between untreated and MP-treated groups. Finally, the analysis of changes in percent free activity in this manner (Fig. 3) demonstrated a lack of a significant effect of MP treatment on this parameter at progressively diminishing levels of MBF.

In conclusion, we have attempted to monitor as many variables as possible (particularly MBF) to compare ischemia-induced changes in lysosomes, microsomal RINCR, and proteins. Using these biochemical parameters we have not been able to identify, after 2 hours of ischemia, a significant efficacious effect due to prior treatment with methylprednisolone. Thus, if this experimental approach can be considered to be relevant to acute clinical myocardial ischemia, the above biochemical evidence would not support treatment of early myocardial ischemia with methylprednisolone.

Addendum

While this manuscript was under review, other work was published as an abstract30 stating that “after 1–2 hr. of ischemia, however, steroid-protected myocytes deteriorated and the biochemical activity and anatomical distribution of cathepsin D was indistinguishable from untreated hearts.” These authors (Decker et al.) reported a beneficial effect at 30–45 minutes of treatment with methylprednisolone (50 mg/kg) in rabbit hearts. This work is in press in the Journal of Clinical Investigation.

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