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R S Decher, ... , J T Dingle, K Wildenthal

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Research Article

Occlusion of the circumflex coronary artery induced a profound redistribution in ischemic rabbit myocardium of several lysosomal acid hydrolases, including cathepsin D, B-acetylglycosaminidase, and acid phosphatase. 30-45 min after ligation non-sedimentable cathepsin D activity rose from 36% of the total activity to 42-48%, and in immunohistochemical preparations cathepsin D appeared to have diffused from lysosomes into the cytosol of injured cells. A pharmacologic dose of methylprednisolone (50mg/kg) significantly delayed the subcellular redistribution of cathepsin D and the other hydrolases in ischemic heart. Thus, in treated hearts the nonsedimentable activity of cathepsin D rose to only 38% after 30 min of ischemia and 42% after 45 min (P is less than 0.05 compared to untreated ischemia at each time). Similarly, unlike untreated hearts, no evidence of enzyme diffusion from lysosomes could be demonstrated immunohistochemically in corticosteroid-treated ischemic hearts for over 45 min. After 1-2 h of ischemia, however, steroid-protected myocytes deteriorated and the biochemical activity and anatomical distribution of cathepsin D were indistinguishable from untreated ischemic hearts. This study demonstrates that corticosteroid pretreatment does not prevent alterations in cardiac lysosomes during severe ischemia indefinitely, but does delay their development significantly.

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Influence of Methylprednisolone on the Sequential Redistribution of Cathepsin D and other Lysosomal Enzymes during Myocardial Ischemia in Rabbits

ROBERT S. DECKER, A. ROBIN POOLE, J. T. DINGLE, and KERN WILDENTHAL, *The Pauline and Adolph Weinberger Laboratory for Cardiopulmonary Research, Departments of Cell Biology, Physiology, and Internal Medicine, The University of Texas Health Science Center at Dallas, Dallas, Texas 75235, and Tissue Physiology Department, Strangeways Research Laboratory, Cambridge, England*

ABSTRACT Occlusion of the circumflex coronary artery induced a profound redistribution in ischemic rabbit myocardium of several lysosomal acid hydrolases, including cathepsin D, B-acetylglucosaminidase, and acid phosphatase. 30–45 min after ligation non-sedimentable cathepsin D activity rose from 36% of the total activity to 42–48%, and in immunohistochemical preparations cathepsin D appeared to have diffused from lysosomes into the cytosol of injured cells. A pharmacologic dose of methylprednisolone (50 mg/kg) significantly delayed the subcellular redistribution of cathepsin D and the other hydrolases in ischemic heart. Thus, in treated hearts the nonsedimentable activity of cathepsin D rose to only 38% after 30 min of ischemia and 42% after 45 min ($P < 0.05$ compared to untreated ischemia at each time). Similarly, unlike untreated hearts, no evidence of enzyme diffusion from lysosomes could be demonstrated immunohistochemically in corticosteroid-treated ischemic hearts for over 45 min. After 1–2 h of ischemia, however, steroid-protected myocytes deteriorated and the biochemical activity and anatomical distribution of cathepsin D were indistinguishable from untreated ischemic hearts. This study demonstrates that corticosteroid pretreatment does not prevent alterations in cardiac lysosomes during severe ischemia indefinitely, but does delay their development significantly.

Dr. Poole is presently Director, Joint Diseases Laboratory, Shriner's Hospital for Crippled Children, Montreal, Quebec, Canada.

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INTRODUCTION

Synthetic corticosteroids have been reported to attenuate the subcellular damage that normally accompanies myocardial ischemia or anoxia in a variety of animal model systems (1–6). The thrust of many of these investigations has been directed at elucidating whether the “membrane-stabilizing” properties of steroids (7) are important in providing the reported protection. Several workers (2, 3, 8–10) have suggested that such membrane stabilization might produce diminished myocardial cell damage during ischemia or hypoxia in part by restricting the subcellular release of lysosomal degradative enzymes into the cytosol.

Other studies, equally carefully performed, have failed to disclose any protective effect of corticosteroids on the function, structure, or lysosomal properties of ischemic or hypoxic hearts (11–16). The explanation for these apparent discrepancies has remained unclear. One possible reason for confusion is that most studies have evaluated only a single time-point after initiation of ischemia in a single experimental model, and considerable differences have existed in the various studies between the times studied as well as in the exact nature and degree of the stress imposed. Accordingly, in the present experiments we have sought to define sequential changes in lysosomal properties at multiple times after coronary occlusion in a previously-described animal model (17) that reproducibly creates severe, rapidly progressive ischemic necrosis. We have focused special attention on early changes, during the period when damage is not yet irreversible. Analysis of these early changes, which may be subtle, has been

facilitated by the use of a newly-available procedure for defining the anatomical distribution of lysosomal cathepsin D by immunohistochemical techniques (17-19), as well as by the use of conventional analyses of the biochemical distribution of several lysosomal hydrolases.

METHODS

New Zealand white male rabbits, aged 4-6 mo (body wt, 2.5-3.0 kg), were fed Wayne rabbit chow (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.) ad libitum. The rabbits were anesthetized with pentobarbital sodium (30 mg/kg), ventilated with a Harvard respirator (Harvard Apparatus Co., Inc., Millis, Mass.), and subjected to thoracotomy. Blood-gas partial pressures and pH were maintained within normal ranges throughout the experiments. 30 min before coronary artery occlusion, 50 mg/kg body weight of methylprednisolone (Solu-Medrol, Upjohn, Inc., Kalamazoo, Mich.) or an equivalent volume of saline was administered slowly through a cannulated ear vein. As described previously (17) a 3-0 silk ligature was positioned around the circumflex branch of the left coronary artery. The artery was ligated for up to 2 h and the ischemic area, which had sharply defined borders, was located visually.

At 0, 30, 45, 60, 90 or 120 min, after arterial occlusion, the heart was excised and placed in 0.9% (wt/vol) NaCl (4°C). Groups of 6-10 rabbits were tested with and without methylprednisolone at each time period. Small sections of transmural ischemic tissue from the center of the cyanotic area supplied by the ligated artery were rapidly removed. Samples of distant, presumably nonischemic ventricle that appeared grossly normal were removed simultaneously.

Biochemistry. Tissue samples were placed in chilled 0.25 M KCl + 1 mM EDTA buffered to pH 7.4 with 50 mM Tris. The tissue slices (always maintained at 4°C) were minced and then homogenized for 30 s with a Willems Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, N. Y.). The homogenates were subsequently assayed for cathepsin D; N-acetyl- β , D-glucosaminidase; and acid phosphatase (all of which are localized at least partially in lysosomes in heart). For measurements of total enzyme activities, the tissue was homogenized vigorously in the presence of Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) to disrupt cells and organelles maximally. Cellular debris was sedimented at 350 g for 5 min and assays were performed on the supernate. For measurements of sedimentable vs. nonsedimentable enzyme activity, hearts were homogenized gently for 30 s in buffered KCl lacking Triton to retain intact lysosomes insofar as possible. After initial centrifugation at 350 g for 5 min to remove undisrupted cells, nuclei, and debris, the supernate was re-centrifuged at 40,000 g for 20 min. The supernate from this second centrifugation was assayed for enzyme activity (termed "nonsedimentable" activity); the pellet was rehomogenized vigorously in a solution containing 0.1% Triton X-100 and also assayed for enzyme activity (termed "sedimentable" activity). The ratio of nonsedimentable to total (i.e., "sedimentable" plus "nonsedimentable") activity was calculated as an index of lysosomal fragility and/or the presence of enzyme not bound to lysosomes. Enzyme activities were assayed by modifications of the methods of Barrett (20) as described previously (18). Briefly, cathepsin D activity was assayed by measuring trichloroacetic-acid-soluble Folin-reactive products after incubation with purified hemoglobin at pH 3.2 and 45°C (Barrett's method II); glucosaminidase, by measuring the amount of nitrophenol cleaved from *p*-nitrophenyl- β -acetyl-D-glucosaminide at pH 4.3 and 37°C; and acid phosphatase, by measuring the amount of nitrophenol cleaved from *p*-nitro-

phenyl-phosphate at pH 4.5 and 37°C after inhibition of non-lysosomal phosphatases with sodium acetate. Protein was measured by the technique of Lowry et al. (21). Analyses of statistical significance between ischemic and nonischemic tissue samples from the same hearts were made with Student's *t* test for paired data. Comparisons between different hearts were made with Student's *t* test for unpaired data with age-matched groups that had been operated on and assayed at the same times; this was necessary because of slight day-to-day variations in the assays and, especially, because of marked differences in the activities and distribution of cardiac lysosomal enzymes, especially cathepsin D, among animals of various ages (22).

Immunohistochemistry. For studies of the morphological location of cathepsin D, small sections of subendocardial ischemic and nonischemic left ventricular tissue were prepared for immunohistochemical localization as described in detail previously (17, 18). The tissue was immersed in a mixture of 7% (wt/vol) gelatin and 150 mM NaCl and immediately frozen in liquid nitrogen. 5- μ m-thick frozen sections were cut, fixed with 4% (wt/vol) formaldehyde, and washed with phosphate-buffered saline plus 5 mM cysteine. Test sections were exposed to sheep anti-(rabbit cathepsin D) Fab' in the presence of 5 mM cysteine for 1 h. The specific sheep antiserum Fab', which remained bound to cathepsin D in the tissue slices, was then itself stained by exposure of the section to fluoresceinated pig anti-(sheep Fab')Fab'. Those sites in the cell where cathepsin D was present could thus be identified microscopically from their specific fluorescent staining. Control sections for nonspecific staining were prepared by exposing the tissue slices first to a Fab' preparation from nonimmune sheep rather than from the sheep in which the antiserum against rabbit cathepsin D had been raised. All control sections displayed only weak diffuse cytoplasmic staining after subsequent treatment with fluoresceinated pig anti-(sheep Fab')Fab', with no evidence of particulate nonspecific staining (17).

RESULTS

Lysosomal acid hydrolase activities. As reported previously (19), the total specific activities of cathepsin D; N-acetyl- β , D-glucosaminidase; and acid phosphatase remained unchanged in saline-treated hearts throughout the 2-h ischemic period. Similarly, specific activities of the acid hydrolases were not altered throughout the experiment in both ischemic and nonischemic sections of hearts pretreated with methylprednisolone.

In contrast, there were marked changes in the distribution of the lysosomal enzymes within the tissue homogenates. In hearts untreated with methylprednisolone, the proportions of total cathepsin D, glucosaminidase, and acid phosphatase activities that were recovered in the nonsedimentable fraction were significantly increased by 30 min in ischemic tissue as compared to nonischemic tissue in the same hearts (Table I). The redistribution progressed over the entire 2-h period for cathepsin D, but peaked by 45-60 min for glucosaminidase and acid phosphatase.

A single pharmacological dose of methylprednisolone administered 30 min before ligation of the circumflex artery had no effect on the distribution of ly-

TABLE I
Influence of Coronary Artery Occlusion on Nonsedimentable Cathepsin D, N-Acetyl-β-D-Glucosaminidase, and Acid Phosphatase Activities in Homogenates of Saline and Methylprednisolone-Treated Myocardium

Time	Saline-treated hearts			Methylprednisolone-treated hearts		
	Non-ischemic	Ischemic	Difference ischemic minus Nonischemic	Non-ischemic	Ischemic	Difference ischemic minus Nonischemic
<i>min</i>						
Cathepsin D						
30	35±1.8	42±2.5	7±1.6*	36±2.6	38±2.9	†2±1.2
45	36±3.8	48±1.9	12±2.6*	39±4.9	42±4.9	†3±3.1
60	37±2.7	53±3.7	16±2.0*	34±2.5	53±2.2	18±2.8*
90	34±2.4	52±2.8	18±2.8*	35±1.5	51±3.7	16±3.3*
120	45±4.1	65±1.7	20±3.2*	41±2.7	61±2.0	20±2.8*
N-Acetyl-β-D-Glucosaminidase						
30	57±2.9	69±2.4	12±1.8*	56±2.1	61±3.3	†5±2.5
45	60±3.8	77±4.2	17±4.3*	65±5.1	71±3.3	†6±2.8
60	49±2.0	66±3.4	17±3.2*	53±3.2	70±1.9	17±2.5*
90	51±6.5	65±2.3	14±1.1*	55±3.0	66±3.4	11±2.7*
120	56±3.5	71±1.6	15±3.8*	55±2.1	71±2.7	16±3.0*
Acid phosphatase						
30	81±1.6	86±0.7	5±1.3*	81±1.2	83±2.0	†2±1.8
45	81±2.4	87±1.1	6±2.3*	82±1.7	84±1.9	†2±1.5
60	75±1.4	83±1.6	8±0.6*	78±1.3	85±0.8	7±1.2*
90	72±0.9	78±0.9	6±0.6*	74±1.1	79±1.0	5±1.6*
120	76±2.2	81±0.9	5±1.9*	76±0.9	80±1.2	4±1.6*

Nonsedimentable activity is expressed as a percentage of total (nonsedimentable plus sedimentable) activity and calculated as the mean ± 1 SEM of 6–10 paired experiments.

* $P < 0.05$, by Student's paired t tests, analyzing differences between ischemic and nonischemic tissue in the same hearts.

† $P < 0.05$, by Student's group t tests analyzing differences between values for enzyme redistribution (ischemic minus nonischemic) in methylprednisolone-treated and saline-treated hearts at the same times after coronary occlusion.

lysosomal enzymes in nonischemic tissue (Table I). The pattern of changes induced by ischemia was considerably different in steroid-treated hearts, however. Redistribution of all enzyme activities into the nonsedimentable fraction was significantly less at 30 and 45 min in treated hearts than in those that had not received methylprednisolone (Table I). But after 1 h, ischemia-induced increases in nonsedimentable enzyme activities were as great after steroids as untreated hearts (Table I), indicating that the lysosomal stabilization, as observed biochemically, was only a transient phenomenon.

Immunohistochemical localization of cathepsin D. Because variations in sedimentable and nonsedimentable lysosomal activities during ischemia might reflect differences in the physical fragility of lysosomal organelles and their artifactual rupture during homogenization rather than actual anatomical translocation of

enzyme in vivo, implementation of an immunohistochemical technique was used to observe directly any changes in the subcellular distribution of cathepsin D molecules within the cells. Nonischemic myocytes, both steroid-treated and control, were characterized at all times by the appearance of discrete particles of cathepsin-D-staining material, primarily in the paranuclear region (Fig. 1a). These organelles were of the size and distribution typical of myocardial secondary lysosomes (17, 18). Diffuse, nonparticulate, background staining was minimal in all nonischemic myocardium.

As early as 30 min after coronary artery occlusion, a redistribution of cathepsin-D-positive immunofluorescence was apparent in the paranuclear regions of many ischemic myocytes. Unlike the particulate staining that characterized nonischemic cells (Fig. 1a), vivid patches of hazy, halo-like fluorescence (Fig. 1c) could

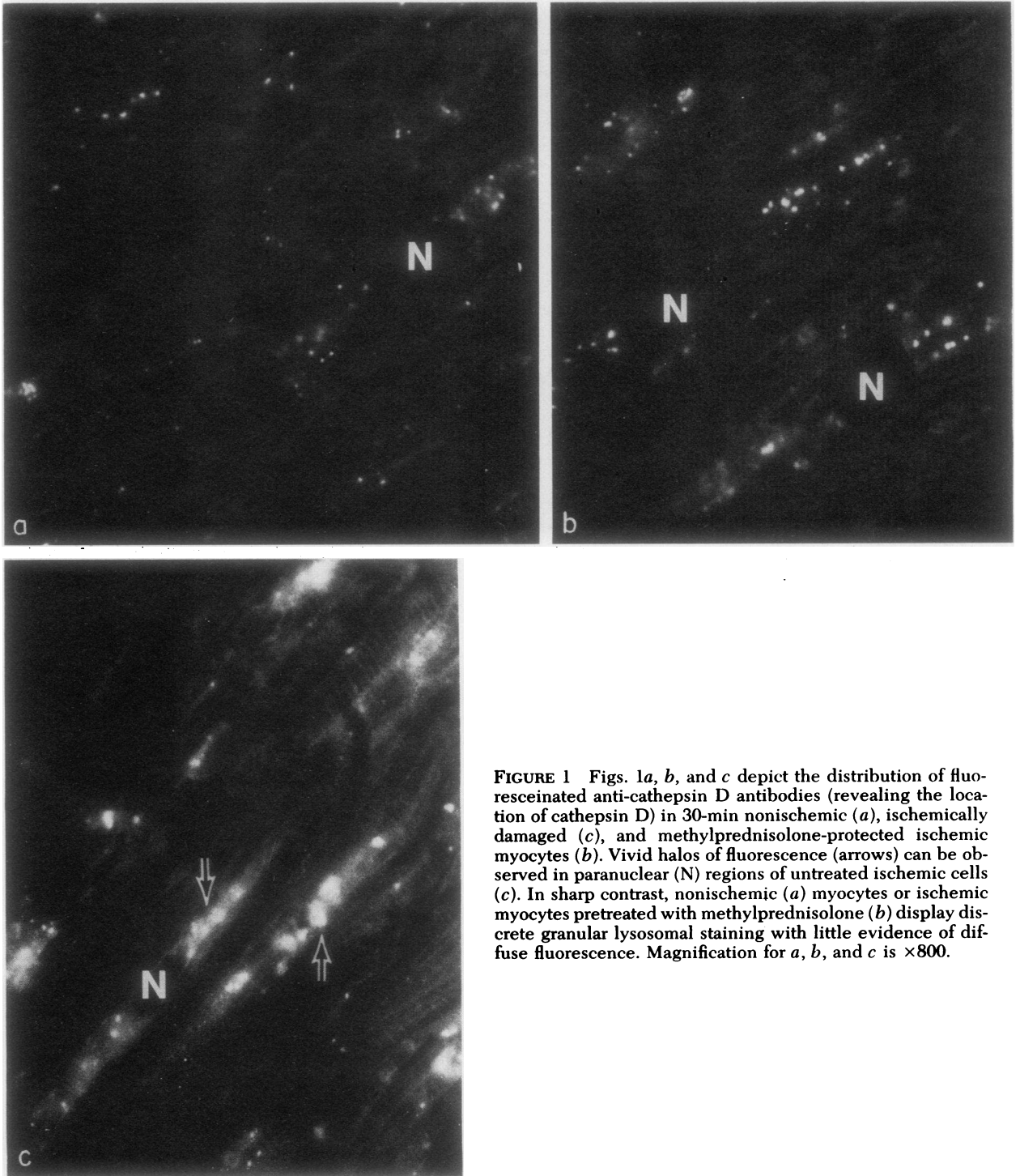


FIGURE 1 Figs. 1*a*, *b*, and *c* depict the distribution of fluoresceinated anti-cathepsin D antibodies (revealing the location of cathepsin D) in 30-min nonischemic (*a*), ischemically damaged (*c*), and methylprednisolone-protected ischemic myocytes (*b*). Vivid halos of fluorescence (arrows) can be observed in paranuclear (N) regions of untreated ischemic cells (*c*). In sharp contrast, nonischemic (*a*) myocytes or ischemic myocytes pretreated with methylprednisolone (*b*) display discrete granular lysosomal staining with little evidence of diffuse fluorescence. Magnification for *a*, *b*, and *c* is $\times 800$.

be observed at the nuclear poles of many ischemically injured cells, suggesting diffusion of enzyme from organelles into the surrounding cytosol. Similar regions of ischemic myocardium obtained from rabbits administered methylprednisolone disclosed no fluorescent

patches; rather, cathepsin-D-positive staining remained sharply confined to lysosomes (Fig. 1*b*). Lengthening the ischemic episode to 45 min increased the number of fluorescent patches that were visualized in ischemic myocytes of untreated rabbits (Fig. 2*b*).

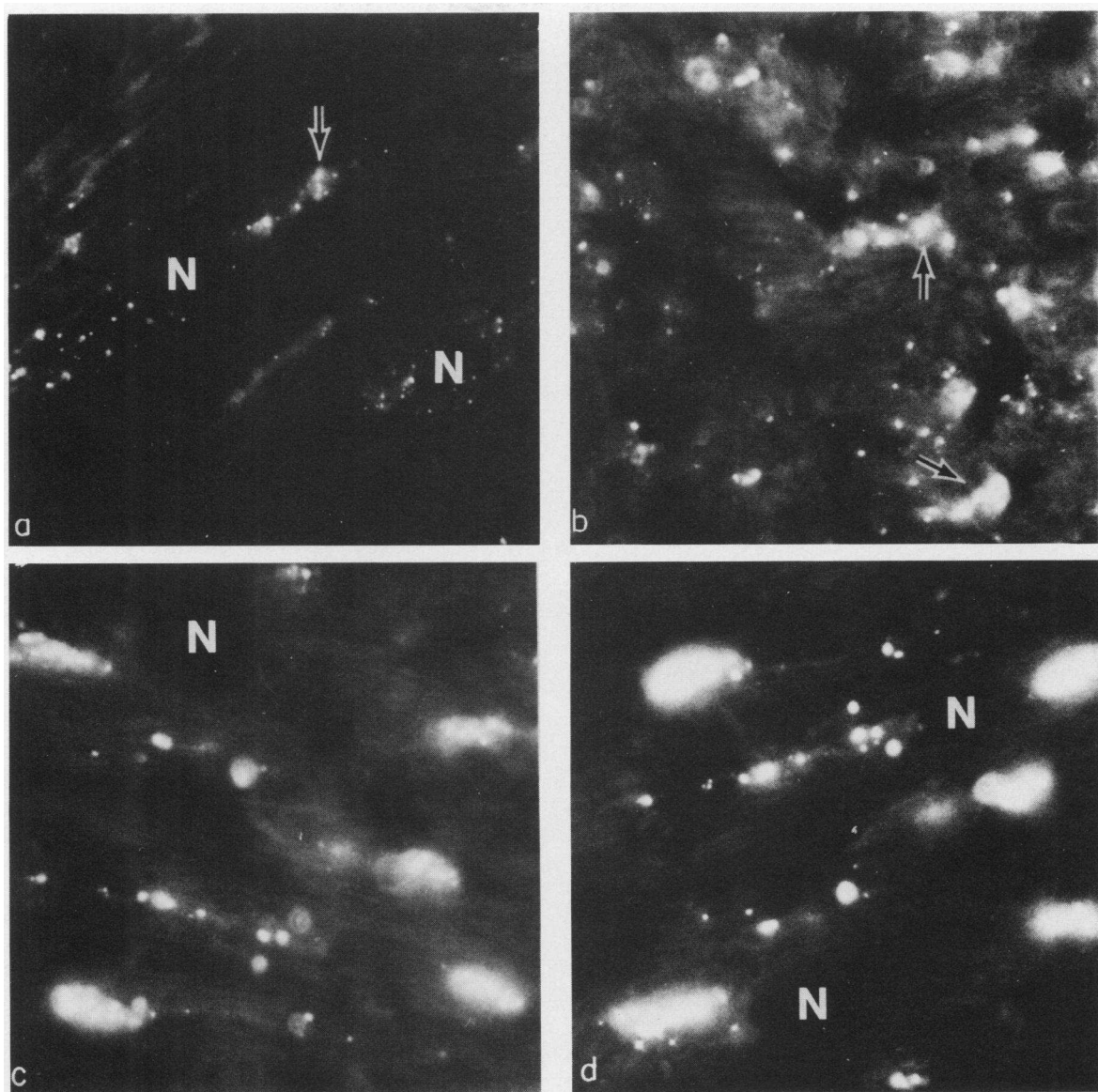


FIGURE 2 Figs. 2*a* and *b* illustrate the localization of anti-cathepsin D antibodies in 45-min methylprednisolone-treated (*a*) and untreated (*b*) ischemic myocytes. Many intense halos of fluorescence and enlarged lysosomes (arrows) are apparent in the injured, untreated myocytes (*b*). Methylprednisolone-treated ischemic myocytes (*a*), like nonischemic myocytes, exhibit primarily granular staining around nuclei (N); rarely, a fluorescent halo (arrow) is visible. Figs. 2*c* and *d* reveal brilliant fluorescent halos of cathepsin D staining in 60-min methylprednisolone-treated (*c*) and untreated (*d*) ischemically injured myocytes. In both tissues, the cells display fluorescent patches composed of large lysosomes encompassed by diffusely stained halos. Magnification for Figs. 2*a*, *b*, *c*, and *d* is $\times 800$.

In contrast, the distribution of cathepsin D immunofluorescence at 45 min remained particulate in methylprednisolone-pretreated ischemic cells (Fig. 2*a*), just as in nonischemic tissue.

By 60 min the intensity of the immunofluorescent aggregates reached a maximum in ischemic myocytes

(Fig. 2*d*). So luminous was the fluorescence that halos of cathepsin D antibody obscured many of the secondary lysosomes, most of which seemed to be losing their discrete boundaries. Moreover, after 60 min ischemic myocardium that had been pretreated with methylprednisolone (Fig. 2*c*) had at last begun to display the

typical perilyosomal halos of cathepsin D staining that were apparent 30 min earlier in many untreated myocytes (Fig. 1c).

During the succeeding hour, cathepsin D staining appeared identical in ischemic myocytes from steroid-treated and untreated hearts. After 90 min of occlusion, instead of the unique fluorescent halos observed earlier, cathepsin D was distributed linearly along the myocytes, suggesting that the enzyme molecules were diffusing away from the paranuclear lysosomal aggregates (Figs. 3a and b); in frozen sections that displayed myofibrillar cross-striation, fluorescently stained material could be observed among the myofibrils (Fig. 3b). By 2 h after coronary artery ligation, sections of untreated and methylprednisolone-treated ischemic myocytes revealed few fluorescent granules and instead exhibited enhanced cytoplasmic staining (Fig. 3c).

DISCUSSION

The notion that glucocorticoids are effective lysosomal stabilizers arose from studies that demonstrated that, in addition to retarding the access of substrates to lysosomal enzymes, steroids were effective in inhibiting the intracellular and extracellular release of these enzymes (8). More recently, Weissmann (23) has demonstrated that many natural and synthetic glucocorticoids also prevent the fusion of lysosomal membranes with the plasmalemma in phagocytically active neutrophils. Together such steroid-membrane interactions may "stabilize" cell membranes by interfering with intracellular membrane flow and by limiting the movement of macromolecules housed within membrane-bound organelles, thereby inhibiting intracellular release of lytic enzymes.

The hypothesis that lysosomal disruption may contribute to myocellular damage during ischemia and related conditions and that, therefore, stabilization of lysosomes by corticosteroids might prevent or reduce injury in ischemic tissue has been discussed widely in recent years (2, 3, 8-10, 17, 24-26). Several studies have provided evidence in support of a beneficial role for steroids in preventing lysosomal abnormalities and reducing ischemic necrosis (2, 3, 8-10), but still other experiments have failed to disclose any beneficial lysosomal effects of steroids in ischemia (14, 16). The present experiments may serve to resolve some of the apparent discrepancies. Thus, with the use of a sensitive immunohistochemical marker of a major myocytic lysosomal hydrolase, as well as conventional biochemical indices of lysosomal stability, it was possible to demonstrate that a pharmacological dose of methylprednisolone causes a definite inhibition of lysosomal labilization in severely ischemic rabbit myocardium at 30 and 45 min after coronary ligation. By 1-2 h, however, no differences could be observed in the lysosomal properties of treated and untreated ischemic

tissue. A similar delay (but not prevention) in the ultrastructural signs of irreversible necrosis (27, 28) was observed in these same hearts (29). On the basis of previous studies (2), it seems unlikely that any of the protective influence of methylprednisolone treatment is mediated by the vehicle used rather than by the steroid itself.

Thus, pretreatment with steroids can preserve lysosomal integrity in severely ischemic hearts, but this preservation is only transient. In our experimental model, in which the tissue tested has incurred a consistent reduction in coronary flow of more than 85% as measured by radioactive microsphere distribution (unpublished data), the period of apparent lysosomal protection is <1 h. It remains possible, of course, that the protective effects of steroids might be relatively greater and last longer in areas of less severe ischemia. Nevertheless, it seems likely that when blood flow is critically and permanently impaired, methylprednisolone can serve only to delay rather than prevent the subcellular changes that lead to necrosis.

It should be emphasized that neither the present results nor previous ones serve to establish definitely either that lysosomal derangements are causally related to the necrotic process or that any beneficial effect of corticosteroids in delaying necrosis or reducing its extent is necessarily related to their lysosomal stabilizing properties. It remains entirely possible that correlations between changes in lysosomal properties and the development of irreversible injury are coincidental, and that any beneficial results of steroid treatment are mediated by factors independent of lysosomes. Nevertheless, the present results are compatible with a role for lysosomes in contributing to the necrotic process and with a role for the temporary alleviation of that process by steroid-induced membrane stabilization.

Finally, it should be noted that, in addition to whatever beneficial effects steroids may have in delaying membrane damage and cell death, they may prevent institution of repair processes as well (30-32). Because there is also some evidence that the lysosomal alterations that accompany ischemic injury may be more importantly related to the initiation of subcellular repair processes than in the progression of injury per se (33), it remains possible that the ultimate effect of inhibiting normal lysosomal responses could be to reduce the ability of injured myocardium to recover. Extrapolations of these results to the clinical setting (where, in any event, evidence for a beneficial effect of steroids in patient with myocardial infarction is less than clear-cut [34, 35]) should therefore be made only with caution.

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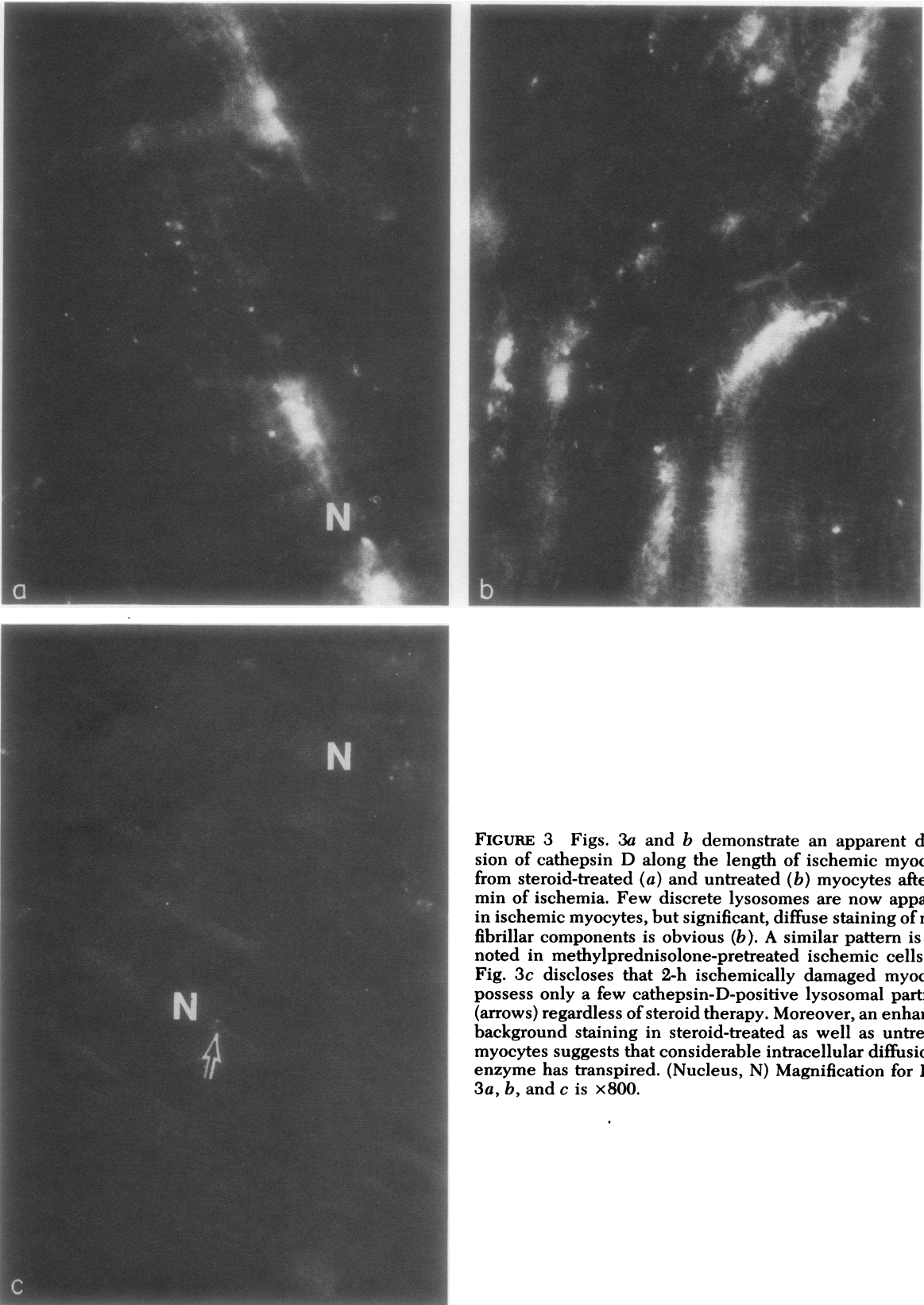


FIGURE 3 Figs. 3*a* and *b* demonstrate an apparent diffusion of cathepsin D along the length of ischemic myocytes from steroid-treated (*a*) and untreated (*b*) myocytes after 90 min of ischemia. Few discrete lysosomes are now apparent in ischemic myocytes, but significant, diffuse staining of myofibrillar components is obvious (*b*). A similar pattern is also noted in methylprednisolone-pretreated ischemic cells (*a*). Fig. 3*c* discloses that 2-h ischemically damaged myocytes possess only a few cathepsin-D-positive lysosomal particles (arrows) regardless of steroid therapy. Moreover, an enhanced background staining in steroid-treated as well as untreated myocytes suggests that considerable intracellular diffusion of enzyme has transpired. (Nucleus, N) Magnification for Figs. 3*a*, *b*, and *c* is $\times 800$.

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