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Inducible Nitric Oxide Synthase Gene Transcription and Protein Activity in the Rat Heart during Endotoxaemia

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Septicaemia leads to an impairment of myocardial contractility in animals and humans. Cytokines released during endotoxaemia are capable of increasing inducible nitric oxide synthase (iNOS) expression *in vitro* in myocytes, endothelial cells and macrophages. The aim of this study was to assess whether iNOS gene transcription occurs in the myocyte *in vivo*. Rats were injected with intraperitoneal endotoxin. Myocardial sections obtained 4, 6 and 8 hours after infection were hybridised with oligonucleotides complementary to iNOS cDNA. Myocardial homogenates were used to measure NOS enzyme activity and to detect iNOS mRNA. Uninfected control animals did not demonstrate myocardial iNOS expression. Myocardium from endotoxaemic animals contained iNOS mRNA and high calcium-independent NOS enzyme activity. *In situ* hybridisation did not localise iNOS to myocytes but to cells located between myocytes. Endotoxaemia leads to iNOS gene transcription and calcium-independent NOS enzyme activity in the rat myocardium. *In situ* hybridisation demonstrates that iNOS is not transcribed by the myocyte *in vivo*.

Septicaemia continues to be an important cause of mortality. Resistant hypotension and multi-organ failure can develop despite treatment with antibiotic and vasopressor therapy. Persistent hypotension associated with a low systemic vascular resistance is a poor prognostic indicator (1). The initial cardiovascular response to the hyperdynamic state of sepsis is a normal to high cardiac output (2). With prolonged sepsis an intrinsic impairment of myocardial function develops which is independent of pre- and afterload (3). This depression of myocardial function has been demonstrated *in-vivo* in septicaemic rabbits (4), dogs (5) and sheep (6). Perfused hearts from septic rats also show diminished left ventricular function (7,8).

In patients septic shock also leads to impaired myocardial contractility with reduced ventricular ejection fractions (9,10). Volunteers treated with endotoxin develop a reduced left ventricular ejection fraction and increased end-systolic and diastolic volumes (3). An impaired myocardial response to β-adrenergic receptor stimulation is also seen in human sepsis (11).

The cause of the intrinsic impairment of myocardial performance seen in endotoxaemia is currently unclear. Coronary blood flow is increased in septic humans (12,13). It has been proposed that this may lead to maldistribution of coronary flow leading to global myocardial ischaemia and depressed cardiac function (14,15). It has also been proposed that a circulating myocardial depressant factor is produced in endotoxaemia (16,17) which inhibits myocardial contractility during infection.

Myocytes isolated from septic rabbits have been shown to have depressed contractility (4). Endotoxin has been shown *in-vitro* to directly inhibit the contractility of isolated guinea-pig myocytes (18). In the same experiments agents that either prevented the synthesis or the effect of nitric oxide (NO) reversed this depression of myocyte contraction produced by endotoxin. Subsequently similar results were obtained *in-vitro* using adult rat myocytes (19). Endotoxin was shown to inhibit rat cardiac myocyte contractility and this inhibition was reversed by inhibitors of NO synthase (NOS).

Thus *in-vitro* studies suggest that endotoxin may directly inhibit myocyte contractility via the induction of NOS. We therefore investigated the endotoxaemic rat

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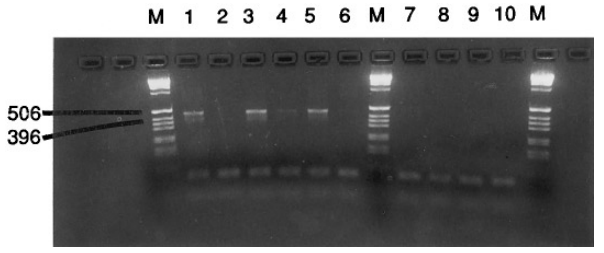


FIG. 1. Post-PCR agarose gel electrophoresis following PCR with oligonucleotide primers specific for rat iNOS. A 464 base pair DNA product is seen in the RT^+ lanes from myocardium of septicaemic rats obtained at 4, 6, and 8 hours (lanes 1, 3, and 5, respectively) after infection. No products are seen in RT^- lanes (lanes 2, 4, and 6) or from control myocardium (lanes 7–10).

model, assessing myocardial gene transcription of iNOS and assessing NOS enzyme activity in myocardial homogenates. In-situ hybridisation was performed to investigate the cell of origin of iNOS.

MATERIALS AND METHODS

Rat endotoxaemia model. Male Wistar rats were injected with intraperitoneal Salmonella endotoxin (4 mg/kg). Control animals were injected with an equivalent volume of intraperitoneal pyrogen free saline. Animals were killed by cervical dislocation 4, 6 and 8 hours after injection. The thoracic cavity was opened and the heart removed immediately. A 3-4 mm size segment of myocardium from the left ventricular wall was excised, avoiding any major blood vessels. The segment was halved for enzyme assay and for in-situ hybridisation. The tissue was immediately stored at −70°C.

Reverse transcriptase polymerase chain reaction (RT-PCR). Extraction of RNA was based on the single step method of Chomczynski and Sacchi (20). Messenger RNA is specifically isolated by binding to Oligo (dT) cellulose. The RNA extract was then incubated with the cloned Moloney Murine Leukaemia Virus Reverse Transcriptase. In order to allow successful reverse transcription to cDNA the reaction included Magnesium Chloride, PCR buffer, dATP, dCTP, dGTP and dTTP, RNA'se Inhibitor and Random Hexamers. The cDNA made following reverse transcription was labelled RT^+. For each RNA sample, this was compared with an incubation without reverse transcriptase (RT^-). Following the PCR reaction, the comparison of RT^+ and RT^- lanes allowed detection of any possible amplification of genomic DNA (figure 1).

The PCR reaction was performed with primers were based on the published sequence of rat iNOS (21). The sequences were: 5′-TGAAGCACATGCAGAAATGAGTACCG-3′ and 5′-CCGTCAGAGGTAACTGTTTACACG-3′, bases 1322-1348 (sense) and 1762-1786 (antisense) respectively of the coding sequence (21), amplifying a 464 base pair product. Primers were incubated with Magnesium Chloride, PCR buffer, AmpliTaq DNA Polymerase and cDNA. All tubes were incubated at 95°C for 35 seconds, 60°C for 2 minutes and 72°C for 2 minutes. The tubes were incubated for 35 cycles and the RT-PCR amplified samples were visualised on 1.5% agarose gels using ethidium bromide. Following DNA extraction sequencing was performed with the primers used in the PCR, dye labelled terminators and Taq cycle sequencing. Reaction products were analysed using a 373A sequencing machine (Applied Biosystems).

In situ hybridisation. Oligonucleotide probes were designed complementary to consensus sequences of mouse and human iNOS cDNA. A three probe cocktail complementary to nucleotide bases 445-480, 1257-1292 and 1842-1879 of the coding sequence of the murine iNOS cDNA was used (22,23). Oligonucleotides were labelled with [α-^{35}S]dATP as described previously (24). Following incubation at 37°C for 8 minutes, the reaction labelling was stopped by addition of 5μl EDTA and denaturing at 70°C for 10 minutes. Sections of myocardium were hybridised as described previously (24). Sections were incubated with 50μl/cm^2 of hybridisation solution and [α-^{35}S]-dATP-labelled oligonucleotide probes. Sections were covered with Sealon-film and incubated in a sealed moist chamber at 43°C for 18-20 hours. Thereafter, Sealon-film was floated off in 1× SSC at room temperature after which the sections were washed twice in 1× SSC at 55°C, twice in 0.5× SSC at 55°C and once in 0.5× SSC at room temperature. Subsequently, sections were dipped in DEPC-treated water, dehydrated through ethanol and autoradiographed for 3-4 weeks. Films were then developed and fixed. Cellular resolution of the hybridisation signal was obtained by dipping of sections into LM-1 Hypercoat emulsion and exposing for 6-8 weeks at 4°C. After developing and fixing, sections were counterstained with cresyl violet (0.1%).

Assay of Ca^{2+}-dependent and Ca^{2+}-independent nitric oxide synthase enzyme activities. Myocardium was snap frozen in liquid nitrogen at the time of cervical dislocation and stored at −70°C. Tissue was subsequently freeze-crushed, homogenised and centrifuged at 20,000g for 20 minutes. The soluble fraction was then used for measurement of calcium-dependent and calcium-independent NOS activities. Assay controls were performed with rat brain and J774 cells for calcium-dependent and calcium-independent activities respectively. Nitric oxide formation in the cytosolic fractions of homogenates of rat myocardium was measured by the formation of radiolabelled [^{14}C]-citrulline from [^{14}C]-L-arginine (25). Duplicate incubations for 10min at 37°C were performed for each sample in the presence or absence of either EGTA (1mM) or EGTA plus N^G-monomethyl-L-arginine (L-NMMA) (1mM each) to determine the level of the Ca^{2+}-dependent and Ca^{2+}-independent NOS activities respectively. The reaction was terminated by the addition of 0.1 vol of 20% (vol:vol) aqueous $HClO_4$. Samples were neutralised by the addition of 0.23 vol of 1.9M aqueous $KHCO_3$, cooled on ice for 5min and centrifuged (10,000g, 2min). [^{14}C]-citrulline in the supernatant was separated from [^{14}C]-arginine by cation-exchange chromatography using AG 50W-X8 resin and quantified by liquid-scintillation counting. Nitric oxide synthase activities are measured in pmol/mg/min.

RESULTS

Nitric oxide synthase activities.

	4 hours	6 hours	8 hours	Control	Control
Ca^{2+}-dep NOS	5.6	3.7	5.1	3.0	3.8
Ca^{2+}-indep NOS	3.8	14.1	11.7	0.9	0.4

Ca^{2+}-dep NOS, calcium dependent NOS activity (pmol/mg/min).
Ca^{2+}-indep NOS, calcium independent NOS activity (pmol/mg/min).

Calcium-dependent NOS activity was detected in all infected and control animals. A low level of background myocardial calcium-independent NOS activity was present in myocardium from control animals. In the septicaemic rat however, there is an increase in the calcium-independent NOS activity within the myocardium. This activity is maximal at 6 hours. There is a 10 fold increase in myocardial calcium-independent NOS activity at 6 and 8 hours after infection.

Reverse transcriptase polymerase chain reaction. A 464 base pair product was detected in myocardium obtained from all 3 infected animals (figure 1). No amplified products were detected in control animals (lanes 7-10) or in RT^- lanes (lanes 2,4,6). The base pair size of the amplified product was that expected from the position of the primers in the rat iNOS sequence. Sequencing confirmed the identity of the PCR product.

In situ hybridisation. Multiple sections were examined from myocardium obtained 6 hours after infection (figures 2-4). An example of the non-specific level of silver staining is shown in figure 2. A background level of silver grains are evenly distributed across the tissue without any cellular localisation. This contrasts with figures 3 and 4 which are examples of localisation of iNOS to cells located between myocytes. There was no suggestion of any localisation to myocytes in any of the sections examined. This appearance was repeated throughout other sections.

DISCUSSION

In-vitro studies have demonstrated that a wide variety of mammalian cell types are capable of expressing iNOS following profound immune or cytokine stimulation (26). It is not clear however, if this cytokine environment exists or is relevant *in-vivo*. *In-vitro* studies have demonstrated that endotoxin or cytokines can stimulate isolated myocytes to produce iNOS (25,27). The contractility of isolated myocytes has been shown to be inhibited by endotoxin in a mechanism involving the induction of myocyte iNOS (18,19). Exogenous NO, produced in endothelial co-culture or by NO gas dissolved in solution, inhibits myocyte contractility (28). Is the production of iNOS by myocytes relevant *in-vivo* during endotoxaemia?

In this investigation we have shown increasing calcium-independent NOS activity in myocardial homogenates from septic rats which is maximal at 6 hours after infection. Gene transcription for iNOS was demonstrated in infected myocardium but was not present in uninfected controls (figure 1). Subsequent in-situ hybridisation did not detect iNOS mRNA in myocytes but localised gene transcription to cells between myocytes (figure 2-4).

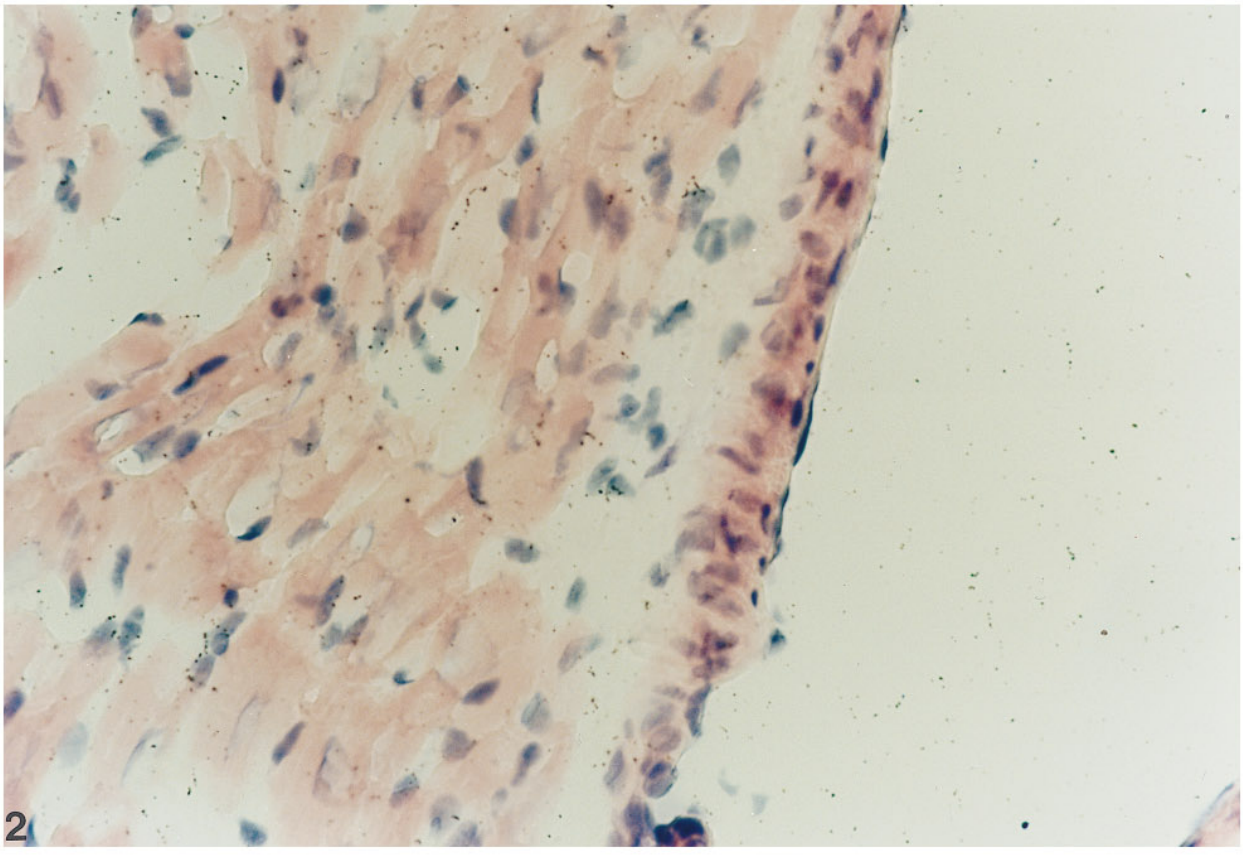
Immunohistochemical techniques have been used to determine the cell of origin of iNOS in the hearts of infected animals. Buttery et al (29) demonstrated iNOS expression in macrophages distributed in both atrial and ventricular myocardium. No staining of myocytes was seen. Balligand et al (30) found iNOS expression in microvascular endothelial cells in-situ in ventricular muscle from lipopolysaccharide treated rats. Thus immunohistochemical studies of rat myocardium have emphasised positive labelling with anti-iNOS in endothelial cells and macrophages but not in myocytes. In this study we did not perform dual labelling with cell type markers and therefore cannot specify the type of cell producing iNOS (figures 3 and 4).

Significant species differences exist for iNOS expression (26). Isolated rodent macrophages have been shown capable of producing iNOS under cytokine stimulation (31). However, demonstration of iNOS activity in human monocytes or macrophages has been difficult (26,32). Some investigators have been unable to show NO production by human monocytes or macrophages (33). There have been some differences noted between human iNOS (hepatocyte) and murine iNOS (macrophage) promoter regions (34). This difference may exist in human macrophage iNOS and may account for difference in stimuli required to enhance high level transcription of iNOS. It therefore remains unclear if macrophage iNOS activity is pathophysiologically relevant in humans.

What may be the functional consequences of this myocardial iNOS production? The coronary microvasculature lies in close proximity to cardiac muscle, most myocytes being within 8μm of their nearest capillary (35,36). Therefore, if NO is produced by microvascular endothelial cells, it will have only a short distance to diffuse to myocytes. Nitric oxide has been shown *in-vitro* to act as a negative inotrope to isolated myocytes (28) and may therefore contribute *in-vivo* to the reduced myocardial contractility seen in endotoxaemia. Functional studies have suggested that large vessel and endocardial endothelial cells may release NO and other substances that may influence myocardial contractility (37,38). Nitric oxide plays an important role in the regulation of myocardial blood flow (39) and vascular smooth muscle cells have been shown capable of iNOS expression (40). Increased microvascular NO production may lead to the inappropriately high coronary blood flow seen in septic shock (12,13).

Myocardial NO may, on the other hand, be acting beneficially in sepsis (41). Nitric oxide has been shown to inhibit platelet adhesion to vascular endothelial cells (42) and may therefore help to reduce platelet thrombosis or platelet consumption in sepsis. Microvascular or macrophage iNOS may act as an antimicrobial agent (43,44) or may regulate neutrophil recruitment by controlling microvascular permeability (45).

Currently inhibitors of NOS which are truly selective for iNOS are not available. In order to further understand the pathological consequences of myocardial iNOS production in septicaemia, functional investigation with selective inhibitors of iNOS will be required. An alternative approach will be the investigation of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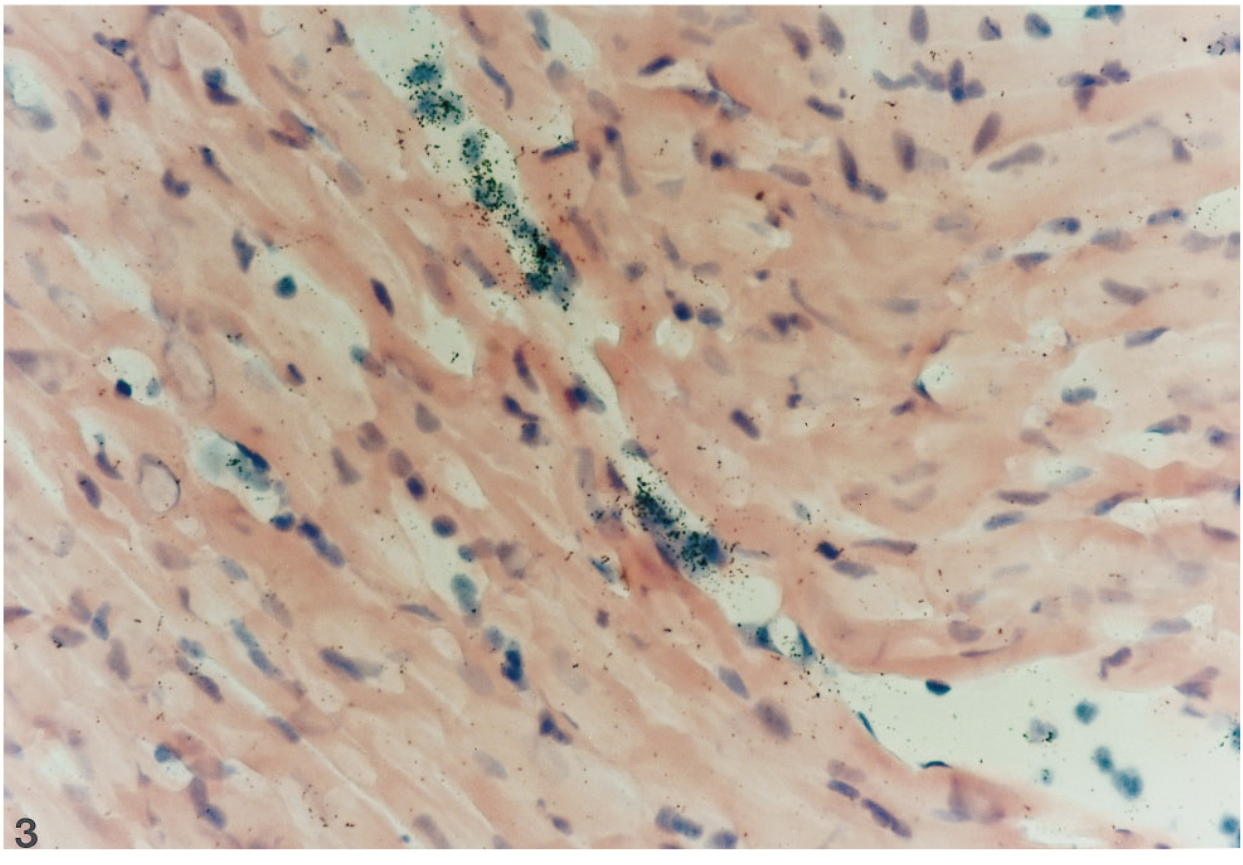
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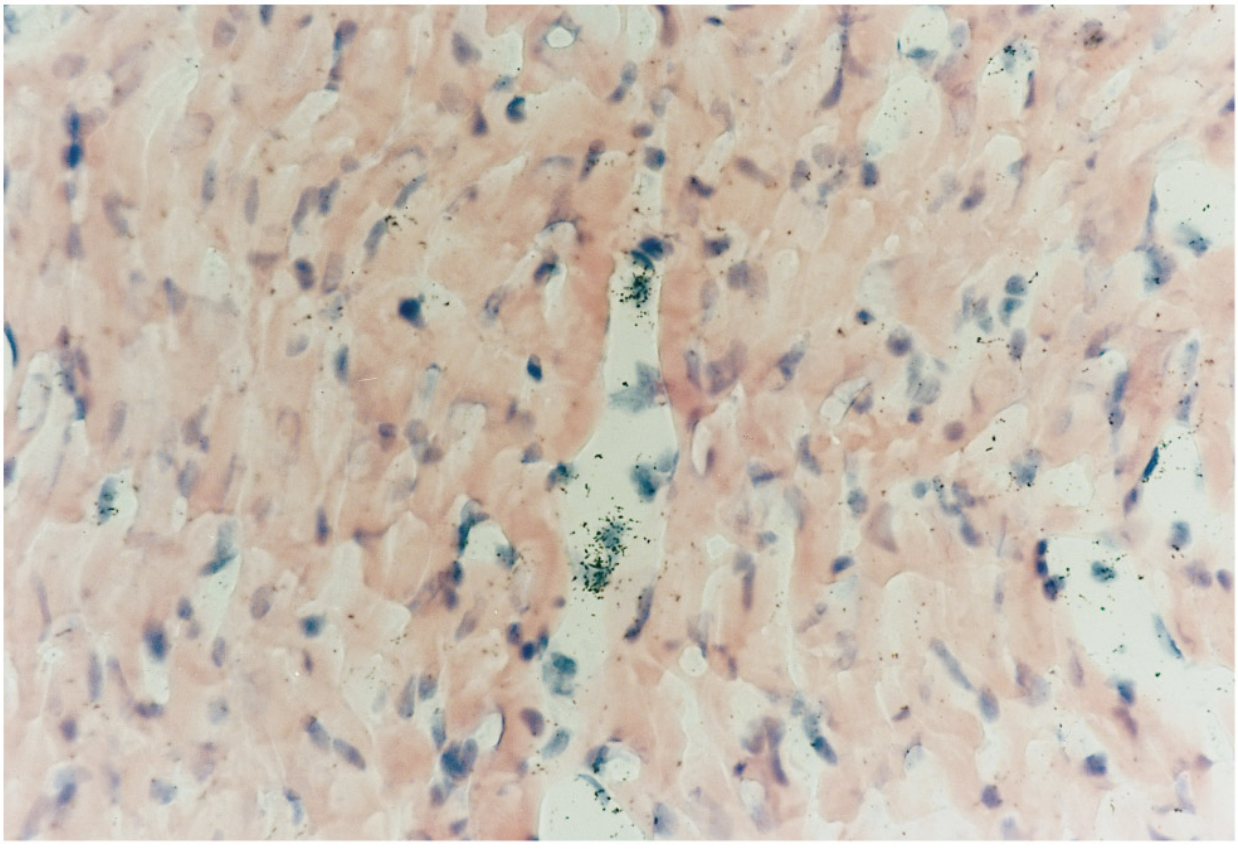

FIGS. 2, 3, 4. Sections of rat myocardium following in-situ hybridisation with oligonucleotide probes complementary to iNOS cDNA. The level of background silver staining is seen in figure 2. Inducible NOS does not localise to myocytes but to cells between myocytes (figures 3 and 4).

"gene knockout" animals which will give insight into the pathophysiological role of inducible nitric oxide synthase (46).

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