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Acute insulin treatment normalizes the resistance to the cardiotoxic effect of isoproterenol in streptozotocin diabetic rats

A morphometric study of isoproterenol induced myocardial fibrosis

O. Gøtzsche

University Institute of Pathology and Second University Clinic of Internal Medicine, University of Aarhus, DK-8000 Aarhus C, Denmark

Summary. The acute effect of insulin treatment on the earlier reported protective effect of streptozotocin diabetes against the cardiotoxic effect of high dosis of isoproterenol (ISO) was investigated in rats. Thirty to 135 min after the injection of crystalline insulin, ISO was given subcutaneously and when ISO induced fibrosis in the myocardium was morphometrically analyzed 7 days later, a highly significant correlation ($r = 0.83$, 2 $p = 0.006$) to the slope of the fall in blood glucose after insulin treatment appeared. The myocardial content of catecholamines was estimated in these 8 day diabetic rats. The norepinephrine content was significantly increased while epinephrine remained unchanged. An enhanced sympathetic nervous system activity with a consequent down regulation of the myocardial β-adrenergic receptors could, therefore, explain this catecholamine resistance. The rapid reversion after insulin treatment excludes the possibility that streptozotocin in itself causes the ISO resistance and points towards a direct insulin effect on myocardial catecholamine sensitivity in diabetic rats. The phenomenon described might elucidate pathogenetic mechanisms behind toxic myocardial cell degeneration and may possibly have relevance for acute cardiovascular complications in diabetic patients.

Key words: Myocardium – Diabetes – Isoproterenol – Insulin effect

Introduction

Experimental studies have shown that the strong β-adrenergic agonist, isoproterenol (ISO) has a marked cardiotoxic effect when given in vivo in large doses (Rona 1963). An excessive calcium influx through the sarcolemma of the myocardial cells, mediated via a β-adrenergic receptor – adenylate cyclase activation, is held responsible for this toxic cell death

Offprint requests to: O. Gøtzsche at the above address

(Fleckenstein 1971; Martorana 1971; Bloom and Davies 1972; Raute-Kreinsen et al. 1977; Katz and Reuter 1979). Clinical and experimental studies have shown a number of modulating factors for this receptor-enzyme coupling (Lefkowitz 1981).

As the release of endogenous catecholamines has been suggested to take part in the propagation of ischaemic lesions in the myocardium (Waldenström et al. 1978), information about the myocardial content and cardiotoxic effect of catecholamines in various conditions disposing to cardiac complications is valuable for the elucidation of the pathogenesis of myocardial cell degeneration. The myocardial catecholamine sensitivity can be registered by histological quantitation of replacement fibrosis 7 days after the in vivo administration of ISO (Gøtzsche 1982) and at the same time this gives indirect information about modulating factors for the calcium permeability of the sarcolemma and the β-adrenergic receptor function in the heart.

One such condition disposing to cardiac complications is diabetis mellitus. Here cardiac deaths and an increased tendency for congestive failure among the patients cannot be explained solely by coronary atherosclerosis (Kannel 1978). The possibility that an abnormal myocardial cell function appears early in the disease has been suggested and the streptozotocin diabetic rat has been established as a model. Based on the observation that insulin protects non-diabetic rabbits against catecholamine induced myocardial lesions (Downing and Lee 1978), a disposition for such lesions could be expected in insulinopaenic, diabetic rats. However, recent experimental studies are inconsistent with this (Fein et al. 1983) and in fact a protective effect of streptozotocin diabetes against the cardiotoxic effect of ISO has recently been reported from our laboratory (Gøtzsche 1982). This unexpected finding of markedly different behaviour of the diabetic myocardial cells could be explained by a decreased calcium permeability of the sarcolomma (Gøtzsche 1983a) and an uncoupling of the myocardial β-adrenergic receptor (Gøtzsche 1983b).

The aim of the present study was to investigate the role of insulin in the pathogenetic mechanism behind the toxic cell death in the myocardium exposed to ISO. At the same time a method for morphometric analysis of ISO-induced fibrosis is described and the tissue content of catecholamines is estimated in order further to clarify the pathogenesis of the ISO resistance of diabetic rat hearts.

Material and methods

Animals. Female Wistar rats weighing 200–300 g were used when they were 3–4 months old. Diabetes was induced by an intraperitoneal injection of streptozotocin (Upjohn, Kalimazoo, MI., USA) (75 mg/kg) and animals with persistent hyperglycaemia (BM Stix with reflectance meter, Boehringer, Mannheim, FRG) but no ketonuria (Ketostix, Ames, Great Britain) were used 8 days later.

Myocardial catecholamine content. After anaesthesia with pentobarbital (30 mg/kg), the animals were opened and 2 ml of blood drawn from v. cava inferior, before the hearts were quickly

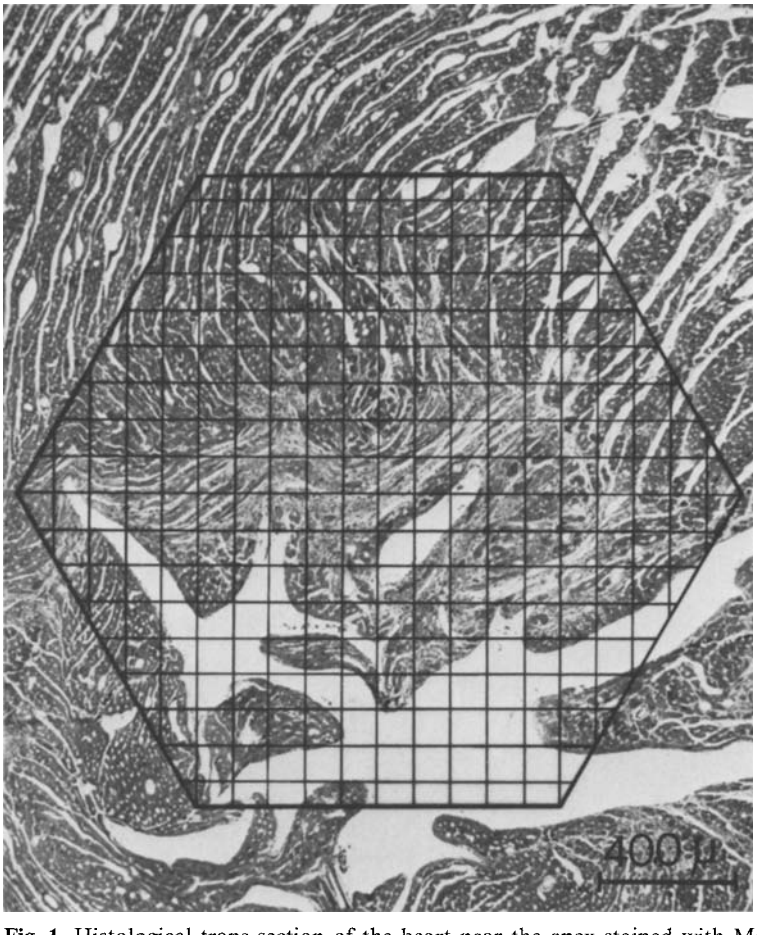

Fig. 1. Histological trans-section of the heart near the apex stained with Masson's trichrome stain and with the grid superposed. The ISO induced fibrosis is seen to be located mainly in the subendocardium

removed, frozen in liquid nitrogen and stored at −70° C until determination for tissue content of epinephrine and norepinephrine. The ventricles were then thawned, weighed and homogenized in cold 0.4 N perchloric acid with the addition of EGTA (0.25 M) and reduced glutathion (0.2 M). After centrifugation the supernatant was assayed for epinephrine and norepinephrine using a double-isotope derivative method (Christensen 1973). Serum levels of sodium and potassium were estimated by atomic absorption spectometry and total calcium colometrically with calcein. The animals used for this part of the study were comparable to the ones that received ISO and that were used for the morphometric analyses of ISO induced fibrosis.

Induction and quantitation of myocardial fibrosis. After the animals were anaesthetized, 30 mg/kg of isoproterenol (Boehringer, Mannheim, dissolved in distilled water with 0,5% sodium pyrosulfite as antioxidant) was injected subcutaneously. Seven days later the animals were again anaesthetized and opened. The hearts were removed and fixed by retrograde perfusion through aorta (2 min, 80 mm Hg) with a formalin buffer (pH 7.4). Postfixation for 7 days in the same buffer was followed by cutting the ventricular part of the hearts into three blocks,

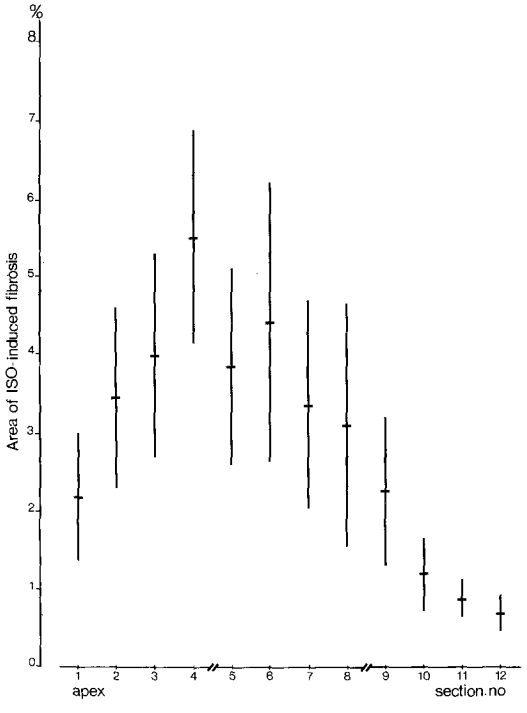

Fig. 2. The extent of ISO induced fibrosis in the 12 sections from the apex towards the atrioventricular plate in hearts from control animals ($n=8$). Bars represent mean ± SEM

and 4 sections (5 μ thick) from each of these blocks at a distance of 200 μ, were stained with Masson's trichrome stain.

ISO induced fibroses was defined as patchy or confluent areas of loose connective tissue replacing normal myocardium and consisting of fibers, fibroblasts and dilated capillaries (Fig. 1). Interstitial and perivascular connective tissue was not included in the measurements and if tangentially cut lamina adventitia from intramyocardial vessels was included, this relatively small error was taken to be equal in all the groups. An earlier comparable study, has, moreover, shown that untreated control rats have negligable connective tissue as defined above in their myocardium (<0.1%) (Gøtzsche 1982).

Morphometric analyses were performed by the same person (O.G.) unaware of the identity of the sections according the principles described earlier (Gøtzsche 1982) and modified according to Gundersen et al. (1980). The magnification of the picture projected to a horizontal screen was 110× and a 255 point grid was used. Each of the six corners of the full-drawn line (Fig. 1) served as reference points (P_r) when hitting tissue (e.g. heart cavity and larger vessel lumina excluded). The ISO-induced myocardial fibrosis was the feature of interest (a) and its volume fraction (V_v) was estimated by counting the intersections of the grid hitting the fibrosis (Pa) within the hexagonal, according to the equation $V_v=\frac{P_a}{P_r}\cdot\frac{6}{255}$. Ninety fields of vision, equidistantly spaced covering approximately 50% of the tissue area, were selected systematically from each heart. Figure 2 shows the mean±SEM of the ISO induced fibrosis in the individual levels of the hearts from 8 control animals. The large scatter was due to variations between the sections within the same animal as well as variation between animals and it can be seen that the fibrosis is mainly located to the mid and lower part of the heart.

In order to test the reproducibility of the morphometry two set of estimations were performed. Firstly the same animal with a volume fraction of 1.27% was measured 5 times, resulting in a coefficient of variation of 4,8%. Secondly, the results from the insulin treated

diabetic animals, ($n=9$) ranging from 0.05–3.38%, was reproduced and the factor of correlation was 0.90.

Insulin treatment. A group of diabetic rats received 4 units of crystalline insulin (Actrapid, NOVO, Copenhagen) intraperitoneally and the blood glucose was followed every 15 min until 30–135 min (range) later when they received the ISO. The fall in blood glucose after insulin differed somewhat and this difference in insulin sensitivity was expressed as the slope of the blood glucose curve.

Statistics. Student t-test for unpaired samples were used except for the differences in ISO induced fibrosis where Wilcoxon's non-parametric test was applied. A $2p=0.05$ level was used as significant.

Results

Blood glucose level and serum electrolytes in controls, diabetic and insulin treated diabetic rats can be seen from Table 1. A significantly reduced ($2p<0.002$) serum potasium is seen in diabetes and as expected acute insulin administration induces a further fall in this variable. Table 2 presents the tissue content (ng/g wet weight) of norepinephrine and epinephrine. Norepinephrine, hold mainly to be located in sympathetic nerve terminals, is seen to be increased in diabetic hearts ($2p<0.05$) while the epinephrine content is unchanged.

The quantitation of ISO induced fibrosis in the experimental groups are seen in Fig. 3. Diabetic rats have a significantly reduced sensitivity to the toxic effect of ISO ($2p<0.01$), and moreover it can be seen that acute

Table 1. Blood glucose and serum electrolytes in control (C), diabetic (D) and insulin treated diabetic rats (D + I). Values are mean ± SEM. Number of animals are given in parenthesis

	Bloodglucose mmol/l	SeCa mmol/l	SeNa mmol/l	SeK llol/l
C (9)	4.8±0.01	2.47±0.04	139±2	5.4±0.4
P		N.S.	N.S.	<0.002
D (11)	17.5±0.8	2.57±0.05	134±2	4.1±0.2
P		N.S.	N.S.	<0.03
D+1 (4)	7.1±0.8	2.60±0.06	135±2	3.4±0.1

Table 2. Norepinephrine and epinephrine in the ventricles of the heart (ng/g wet weight). Values are mean ± SEM

	Norepinephrine ng/g	Epinephrine ng/g
C (9)	624±26	15.2±0.7
P	<0.05	N.S.
D (11)	719±35	14.1±0.6

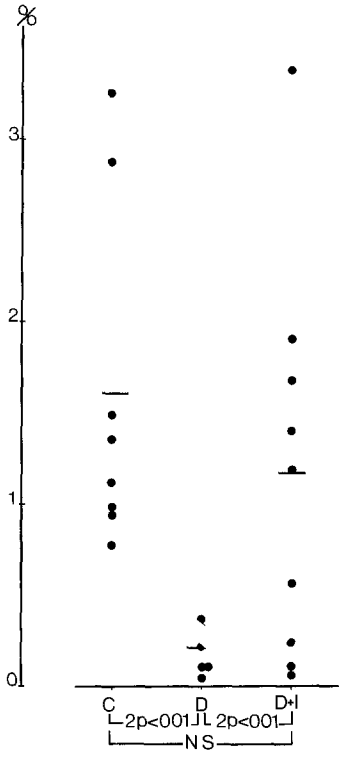

Fig. 3. Volume fraction in per cent of isoproterenol induced fibrosis in the ventricles of the heart. C: control rats, D: diabetic rats, D + I: diabetic rats pretreated with insulin 30–135 min before the administration of isoproterenol. Bars represent mean

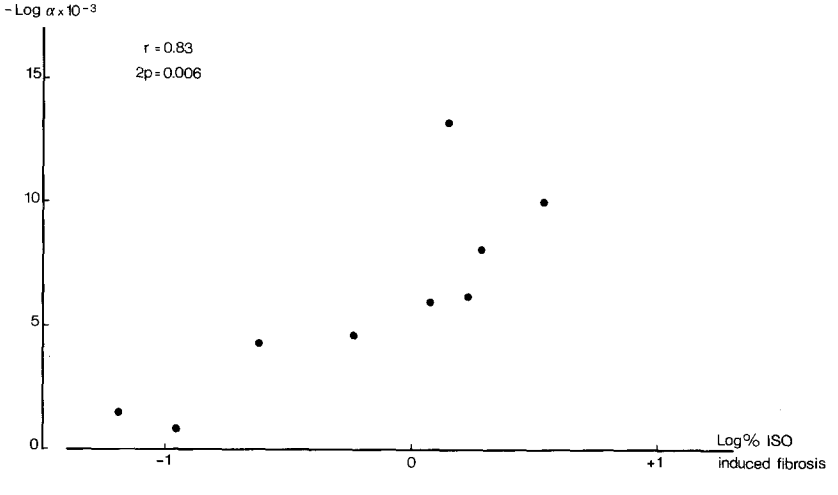

Fig. 4. Correlation between volume fraction of isoproterenol induced fibrosis in the ventricles of the heart and the slope of the fall in blood glucose after insulin treatment (α) in diabetic rats. Both axis have been expressed logarithmically

insulin administration 30–135 min before the injection of ISO, normalizes this response ($2p < 0.01$). The results from this group showed a large scatter. However, when analyzing the insulin effect by determining the slope of the blood glucose fall (α), these values showed a highly significant correlation with the ISO induced fibrosis ($r = 0.83$, $2p = 0.006$, all values logarithmically converted) (Fig. 4). In these animals insulin induced a fall in blood glucose from 15.9 ± 0.8 mmol/l at the time of insulin injection down to 8.1 ± 1.6 mmol/l when ISO was administered.

Discussion

The present study has shown a highly significant correlation between the in vivo insulin effect as judged by the fall in blood glucose in streptozotocin diabetic rats, and the cardiotoxic effect of ISO. This implies that acute metabolic changes in these animals confers dramatic alterations in the catecholamine sensitivity in the heart and excludes the possibility that streptozotocin in itself causes the catecholamine resistance in diabetic rats.

A recent study from another laboratory, using semiquantitative methods for estimating lesions 48 h after the administration of ISO, showed a trend towards desensitization in diabetic rats (Fein 1983). Using exact morphometric methods for measuring the volume fraction of established myocardial lesions, the present study has, however, confirmed our earlier observation that diabetes protects rats against the cardiotoxic effect of ISO.

ISO is a strong β-adrenergic agonist mediating its intracellular effect by stimulating the β-adrenergic receptor – adenylate cyclase system. Abnormalities in this transmembrane signalling process have been suggested in experimental diabetes (Ingebretsen et al. 1981; Williams et al. 1983; Gøtzsche 1983). Short term agonist-induced down regulation of the adrenergic sensitivity in the heart is known to occur (Marsch et al. 1980) and although little is known about the relation between the absolute catecholamine content in the heart and the turnover rate of endogenous catecholamines, the increased tissue level of norepinephrine reported here could be of relevance for the induction of the ISO resistance in these animals. The results are in accordance with the study of Paulsen et al. (1981) also reporting an increase in serum level of norepinephrine in short term diabetic rats. The relation of this enchanced sympathetic nervous system activity in diabetic rats to the observed decreased tissue level of norepinephrine in long term diabetic hearts from humans is unclear (Neubauer and Christensen 1976).

Although administration of sodium of potassium has been shown by Rona et al. to affect the myocardial sensitivity to ISO (Rona 1963) a decreased serum potassium level, as found in the present diabetic rats should be expected to sensitize to and not protect against, the ISO induced myocardial lesions. This observation cannot explain the ISO resistance in the diabetic rat hearts.

In non-diabetic rabbits, insulin has been shown to protect against catecholamine induced myocardial lesions (Downing and Lee 1978). This can-

not, however, be extrapolated to mean that insulinopaenic rats are supersensitive to the toxic effect of strong β-adrenergic stimulation. In fact, if one can extrapolate from differences in species, this implies that insulin has opposing effects on the catecholamine action in control and diabetic hearts.

Excessive calcium influx to the myocardial cells is held to be responsible for the cardiotoxic effects of ISO (Fleckenstein 1971; Hofmann 1977). The present finding that insulin has an acute normalizing effect on the sensitivity to ISO, is consistent with the recent finding from our laboratory that insulin normalizes the decreased myocardial calcium uptake after ISO in isolated perfused diabetic rat hearts (Gøtzsche 1984).

These sudden insulin induced changes in sensitivity to ISO in rats with streptozotocin diabetes, might possibly affect myocardial function and integrity and we suggests that it may be significant in acute cardiovascular complications in diabetes mellitus.

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