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# Succinic acid monomethyl ester protects rat pancreatic islet secretory potential against interleukin-1β (IL-1β) without affecting glutamate decarboxylase expression or nitric oxide production

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**Abstract**

Rat pancreatic islets exposed to interleukin-1β (IL-1β) in the presence of succinic acid monomethyl ester (SAM) have a higher insulin release in response to glucose and higher glucose oxidation rates, as compared to islets exposed to IL-1β alone. These beneficial effects of SAM were not accompanied by any decrease in IL-1β-induced nitric oxide (NO) production nor inhibition of aconitase activity. Moreover, SAM did not increase biosynthesis of glutamate decarboxylase. SAM apparently improves β-cell function mostly by increasing the capacity of these cells to endure NO exposure and partial blockage of the Krebs cycle.

*Key words:* Interleukin-1β; Succinate ester; Nitric oxide; Pancreatic islet; Glutamic acid decarboxylase; Aconitase; Diabetes mellitus

## 1. Introduction

The autoimmune process leading to insulin-dependent diabetes mellitus may evolve for several years before the clinical onset of the disease [1]. During this period, a negative balance between β-cell damage and repair progressively decreases β-cell mass (reviewed in [1,2]). Up to now, most clinical and experimental trials to prevent the outbreak of IDDM involved attempts to arrest the autoimmune assault against the β-cells [3]. It would also be of interest to increase the β-cell capacity to repair itself. Indeed, nutrients such as glucose and amino acids, and the vitamin $B_3$-derived compound nicotinamide, improve β-cell function and survival following cytotoxic damage [2]. However, glucose and α-ketoisocaproic acid also increases the expression of glutamate decarboxylase (GAD) [4–6], a major islet cell autoantigen in IDDM [7]. This could potentially exacerbate the autoimmune assault against the β-cells. Moreover, prolonged exposure of these cells to hyperglycemia may be deleterious to β-cell function [8].

It has previously shown that methyl esters of succinic acid can be taken up and metabolized at the Krebs cycle level by pancreatic β-cells, leading to increased insulin biosynthesis and release [9–12]. Interestingly, these esters also protect pancreatic islets in vivo against the diabetogenic agent streptozotocin [13]. The ability of succinic acid esters to gain direct access to the Krebs cycle, by increasing the influx of both succinate and acetyl residues [12], suggested that these esters may be useful in the treatment of non-insulin dependent diabetes mellitus (NIDDM). Indeed, the alteration of β-cell secretory response to glucose in NIDDM may be due to a site-specific defect in the early steps of glucose metabolism, and these defects could be bypassed by succinate esters [14]. In some experimental models of β-cell damage and dysfunction in IDDM there is also a site-specific impairment in glucose metabolism. Thus, when pancreatic islets are exposed to IL-1β, a cytokine with potential role for β-cell destruction in early IDDM [15], there is generation of nitric oxide (NO) [16–18]. This radical leads to decreased activity of the Krebs cycle enzyme aconitase [17], with consequent decrease in oxidative phosphorylation and β-cell ATP production, impairment in insulin biosynthesis and release, and eventually cell death [19]. Considering that succinate esters enter the Krebs cycle at steps distal to isocitric acid, the final product of aconitase activity, it would be of interest to evaluate if these esters can improve the function of pancreatic islets exposed to IL-β. In the present study we investigated the effects of succinic acid monomethyl ester (SAM) on the IL-1β actions on rat pancreatic islets. Moreover, we also tested

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if SAM leads or not to an increased biosynthesis of the β-cell autoantigen GAD.

## 2. Material and methods

Pancreatic islets were isolated by collagenase digestion from adult male Sprague–Dawley rats bred in a local colony (Biomedical Centre, Uppsala, Sweden). After isolation the islets were cultured free-floating in medium RPMI 1640 containing 11.1 mM glucose and supplemented with 10% (volume/volume) fetal bovine serum [20]. The islets were precultured for 4–6 days under the conditions described above (medium was changed every two days) before exposure to 25 U/ml Il-1β with or without the addition of 10 mM SAM. Human recombinant IL-1β was kindly provided by K. Bendtzen, Laboratory of Medical Immunology, Rigshospitalet, Copenhagen, Denmark. The biological activity of IL-1β was 50 U/ng, as compared with an interim international preparation (NIBSC, London, UK). It has been previously shown in dose–response studies that 10 mM SAM induces a clear stimulation in islet insulin biosynthesis and release [11]. After 24 h exposure to the different agents, the islets were harvested and used for the different experimental procedures.

Insulin release, insulin and nitrite accumulation in the medium, islet DNA and insulin contents were determined as previously described [8,17]. Briefly, insulin release was studied in triplicate groups of 10 islets by a first hour incubation with 1.7 or 2.8 mM glucose. The incubation medium, Krebs–Ringer bicarbonate buffer (KRBH), was then gently removed and replaced by medium containing either 16.7 mM glucose or 2.8 mM glucose + 10 mM SAM and the incubation continued for a second 60-min period. For the determination of [U-$^{14}$C]glucose (293 mCi/mM; Amersham International, UK) oxidation, triplicate groups of 10 islets were transferred to glass vials containing 100 μl KRBH and nonradioactive glucose to a final concentration of 16.7 mM glucose. Islet glucose oxidation was subsequently measured as described in detail elsewhere [8,17]. In these and the above described experiments, the mean of the triplicate observations was considered as one observation. Islet aconitase activity was determined in groups of 150 islets, as previously described [17].

For determination of GAD biosynthesis, groups of 200 islets were cultured for 24 h in the presence of IL-1β and/or SAM, as described above. After this period the islets were washed twice with methionine-free RPMI 1640, and then incubated for 6 h in methionine-free RPMI 1640 containing 10% dialyzed calf serum and [$^{35}$S]methionine (> 1100 Ci/mmol; Amersham International). SAM and/or IL-1β were present during the labelling period at the same concentration as in the previous 24-h culture. After this, the islets were lysed and prepared for immunoprecipitations as described elsewhere [4,6]. Lysates from islets cultured in each of the different experimental conditions were precipitated using two different sera, a rabbit anti-rat MHC class I antiserum (K271), and a sheep anti-rat GAD antiserum (NIMH-1440) reacting against both isoforms of GAD [21]. Immunocomplexes were collected and analysed by fluorography of sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [4,6], or scanned in a phosphor imager (Molecular Dynamics, Sunnyvale, CA) and analysed using the ImageQuant software program (Molecular Dynamics). The results are presented as the ratios between values for GAD and MHC class I.

Data were computed as the mean ± S.E.M. and compared using Student's *t*-test for paired or unpaired samples, as appropriate. When multiple comparisons were performed, the data were analysed by analysis of variance (ANOVA), followed by group comparisons using the Student's *t*-test. The *P* values were corrected for multiple comparisons using the Bonferroni method [22].

## 3. Results

Exposure of rat pancreatic islets to IL-1β induced a 50–70% decrease in both basal (1.7 mM) and glucose (16.7 mM)-stimulated insulin release (Table 1). Islets treated with IL-1β + SAM have significantly higher insulin release in response to glucose than islets treated with IL-1β alone. In line with these observations, islets exposed to IL-1β released less insulin into the culture medium (41% of the values observed in control islets; $P < 0.01$), while islets exposed to IL-1β in the presence of SAM have medium insulin values which were not significantly different from that observed in control (RPMI) or SAM (RPMI + SAM) islets (data not shown). All experimental groups have similar islet insulin and DNA contents (data not shown).

It has been previously suggested that the suppressive effects of IL-1β on rat pancreatic islets are mediated by NO production and subsequent inhibition of the Krebs cycle enzyme aconitase [16–18]. In order to investigate if the protective effects of SAM were related to preventing IL-1β-induced NO production and aconitase inhibition, we measured medium nitrite accumulation and islet aconitase activity (Table 1). As expected, IL-1β induced an increase in medium nitrite accumulation (see below) and a decrease in aconitase activity (Table 1). Control islets (RPMI) and islets exposed to SAM (RPMI + SAM) have undetectable amounts of nitrite in the medium,

Table 1

Insulin release, glucose oxidation and aconitase activity of rat pancreatic islets cultured for 24 h in the presence of IL-1β (25 U/ml) and/or succinic acid monomethyl ester (SAM; 10 mM)

| Culture condition | Insulin release (ng insulin/10 islets·1 h) | | Glucose oxidation (pmol/10 islets·1.5 h) | Aconitase activity (pmol/islet·min) |
|---|---|---|---|---|
| | 1.7 mM gluc | 16.7 mM gluc | | |
| RPMI | 10 ± 2 | 99 ± 6 | 513 ± 62 | 27 ± 3 |
| RPMI + SAM | 11 ± 2 | 129 ± 10 | 471 ± 48 | 31 ± 4 |
| RPMI + IL-1β | 5 ± 1* | 27 ± 4**$^{¥}$ | 103 ± 9**$^{¥}$ | 3 ± 1** |
| RPMI + IL-1β + SAM | 7 ± 2 | 76 ± 10$^{§}$ | 247 ± 10*$^{§}$ | 4 ± 1**$^{§}$ |

Insulin release was measured by incubating the islets in triplicate groups of 10 in KRBH buffer containing 1.7 mM glucose. After 1 h, the medium was exchanged for KRBH supplemented with 16.7 mM glucose. Glucose oxidation rate was measured in triplicate groups of 10 islets at 16.7 mM glucose, and aconitase activity was measured (after sonication) in groups of 150 islets, as described in section 2. Values are means ± S.E.M. of 8–10 separate experiments (insulin release), 6 experiments (glucose oxidation) or 4 experiments (aconitase activity). *$P < 0.05$ and **$P < 0.001$ when compared to RPMI islets, $^{¥}P < 0.01$ when compared to RPMI + IL-1β + SAM islets and $^{§}P < 0.01$ when compared to RPMI + SAM.

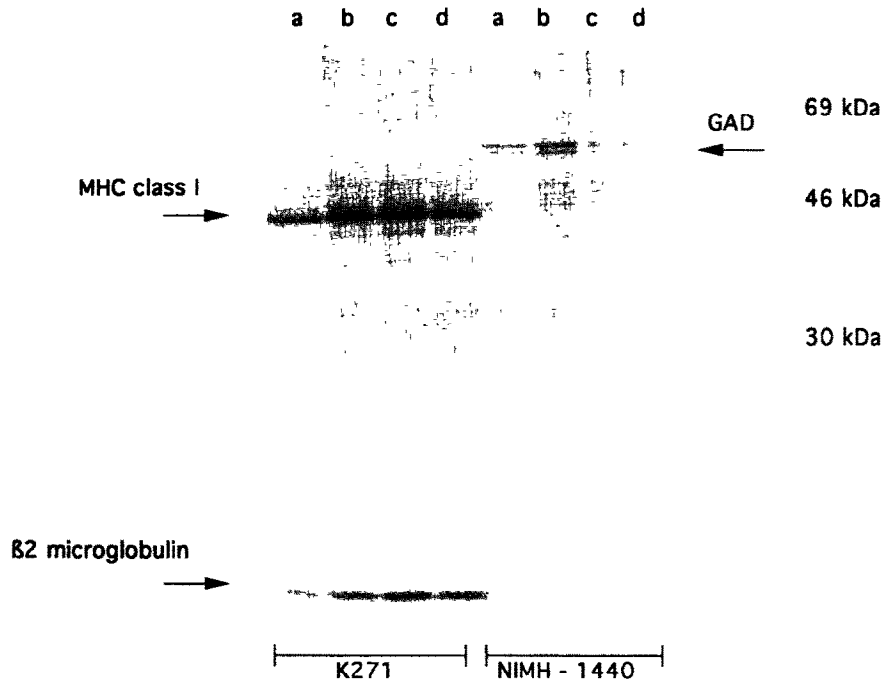


Fig. 1. Fluorography of an SDS-PAGE of immunoprecipitates from rat islets lysates labelled with [$^{35}$S]methionine. In the upper margin, the lanes labelled a represent lysates of islets cultured in the presence of RPMI only, the lanes labelled b islets cultured with RPMI + 10 mM SAM, the lanes labelled c islets cultured with RPMI + 25 U/ml IL-1$\beta$ and the lanes labelled d islets cultured with RPMI + 25 U/ml IL-1$\beta$ + 10 mM SAM. In the lower margin it is indicated the antiserum used in the immunoprecipitation: K271, a rabbit antiserum against the MHC class I, and NIMH-1440, a sheep antiserum against GAD. In the lateral margins the arrows indicate the approximate positions of GAD, the MHC class I heavy chain and $\beta$2 microglobulin, which is coprecipitated with the MHC class I by the K271 antiserum. In the right-hand margin the position of molecular weight markers are depicted.

while islets exposed to IL-1$\beta$ (RPMI + IL-1$\beta$) presented high nitrite values (18.1 ± 2.7 pmol/islet·24 h; mean ± S.E.M.; $n$ = 8), similar to the values observed in islets exposed to IL-1$\beta$ + SAM (RPMI + IL-1$\beta$ + SAM; 20.0 ± 3.5 pmol/islet·24 h; $n$ = 8). Thus, the effects of the cytokine on NO production and aconitase activity were not prevented by SAM. However, SAM partially prevented IL-1$\beta$-induced decrease in islet glucose oxidation (Table 1). Glucose metabolism of IL-1$\beta$ + SAM-treated islets was around 55% of the values observed in islets cultured in the presence of SAM alone, similar to the insulin release data, where values for the IL-1$\beta$ + SAM group were around 60% of the values observed in SAM islets (Table 1). On the other hand, islets exposed to IL-1$\beta$ alone have glucose oxidation and insulin release values which were only 20–30% of the values observed in the respective control islets (Table 1).

When considering agents potentially able to improve $\beta$-cell function in early IDDM it is important to assess if these agents induce an increased biosynthesis and expression of islet autoantigens. To test if these was the case for SAM, we determined the biosynthesis of GAD, an autoantigen in IDDM, in islets exposed to SAM and/or IL-1$\beta$. In four experiments, the immunoprecipitates separated by SDS-PAGE were analysed by fluorography (Fig. 1). By visual inspection, SAM or SAM + IL-1$\beta$ exposure did not increase GAD biosynthesis, as compared respectively to control islets or islets exposed to Il-1. IL-1$\beta$ decreased GAD expression, without affecting HLA class I expression. Quantitative analysis of immunoprecipitable GAD by the phosphor imager (expressed as the ratios between values corresponding to GAD and MHC class I) showed arbitrary values of 0.57 ± 0.10 ($n$ = 4) in control islets, and 0.49 ± 0.07 ($n$ = 4) in islets cultured in the presence of SAM. IL-1$\beta$ induced a marked decrease in GAD biosynthesis (IL-1$\beta$,

Table 2

Insulin release of rat pancreatic islets cultured for 24 h in the presence of IL-1$\beta$ (25 U/ml) and then exposed for 1 h to 16.7 mM glucose or 10 mM SAM + 2.8 mM glucose

| Culture condition | Insulin release (ng insulin/10 islets·1 h) | | |
|---|---|---|---|
| | 2.8 mM gluc | 16.7 mM gluc | 10 mM SAM |
| RPMI | 13 ± 3 | 87 ± 9 | – |
| RPMI + IL-1$\beta$ | 7 ± 1* | 24 ± 2** | – |
| RPMI | 15 ± 3 | – | 73 ± 8 |
| RPMI + IL-1$\beta$ | 8 ± 2 | – | 21 ± 3** |

Insulin release was measured by incubating the islets in groups of 10 in KRBH buffer containing 2.8 mM glucose. After 1 h, the medium was exchanged for KRBH supplemented with either 16.7 mM glucose or 10 mM SAM + 2.8 mM glucose. Values are means ± S.E.M. of 6 separate experiments, each performed in triplicate. *$P$ < 0.05 and **$P$ < 0.001 when compared to respective control islets.

0.22 ± 0.06, $n = 4$; $P < 0.01$ vs. control islets), and this decrease was not counteracted by SAM (IL-1$\beta$ + SAM, 0.19 ± 0.05; $n = 4$; $P < 0.01$ vs. control or SAM-treated islets).

In a final series of experiments, the effects of a short-term (1 h) incubation in the presence of SAM on insulin release by islets exposed for 24 h to IL-1$\beta$ were evaluated (Table 2).As described in Table 1, 24h treatment with IL-1$\beta$ again induced a clear decrease in insulin release both at low and high glucose concentrations (Table 2). A similar defective insulin release was observed when islets were stimulated with 10 mM SAM instead of 16.7 mM glucose. Indeed, in both cases insulin release was 30% of that observed in control islets.

## 4. Discussion

The present data show that SAM partially protects rat pancreatic islets against the suppressive effects of IL-1$\beta$. This conclusion is based on the observations that islets exposed to IL-1$\beta$ in the presence of SAM have higher insulin release in response to glucose, preserved insulin accumulation into the culture medium and higher glucose oxidation rates than islets exposed to IL-1$\beta$ alone. These beneficial effects of SAM were not accompanied by any stimulation of the biosynthesis of GAD, a major autoantigen in early IDDM [7]. This is specially relevant if one considers the potential use of SAM as an adjuvant therapy of early IDDM, aiming at improving $\beta$-cell survival and function in the context of an ongoing autoimmune assault. Indeed, other agents that may stimulate $\beta$-cell function, like glucose, $\alpha$-ketoisocaproic acid and sulfonylureas, have previously been shown to also increase GAD biosynthesis, both in rodent [4,5] and human pancreatic islets [6]. To our knowledge, SAM is the first described agent which improves $\beta$-cell function without increasing GAD expression.

The regulation of GAD expression in $\beta$-cells still remains to be clarified. Available data suggest that GAD activity and expression usually parallels insulin biosynthesis and release [4–6]. In agreement with this, SAM did not increase GAD biosynthesis, insulin release, insulin accumulation into the medium or islet insulin content in the control islets (not exposed to IL-1$\beta$). Moreover, IL-1$\beta$ inhibited in parallel all these parameters. Interestingly, when islets were exposed to both IL-1$\beta$ and SAM there was a clear inhibition of GAD biosynthesis without a similar impairment in insulin release. This suggest that under specific conditions GAD synthesis may be dissociated from insulin synthesis and release. Further characterization of this condition may help to clarify the control of GAD expression in $\beta$-cells.

It has been previously proposed that the cytokine IL-1$\beta$ may be an important mediator of $\beta$-cell damage in early IDDM [15], probably by inducing generation of NO [16–18]. Indeed, rodent insulin producing cells exposed to IL-1$\beta$ express mRNA for the inducible form of nitric oxide synthase [23,24] and produce large amounts of NO [16–18,23,24]. This radical leads to impairment in mitochondrial function, at least in part by blocking the activity of aconitase [17]. IL-1$\beta$ alone does not induce NO production nor inhibits function of human pancreatic islets [25], but when these islets are exposed to combinations of cytokines, namely IL-1$\beta$ + tumor necrosis factor-$\alpha$ + interferon-$\gamma$, there is both induction of NO and impairment in islet function [26,27]. Based on these observations it has been suggested that NO, produced either by invading macrophages or by the $\beta$-cells themselves, may be a final common pathway for $\beta$-cell damage in early IDDM [28,29]. In the present series of experiments, SAM protected the $\beta$-cells without preventing IL-1$\beta$-induced NO production or inhibition of aconitase activity. Thus, the beneficial actions of succinate are probably not related to interference with the initial steps of IL-1$\beta$ effects, such as binding to surface receptors, induction of iNOS mRNA transcription and NO production and blockage of aconitase activity. Indeed, SAM seems to improve $\beta$-cell function mostly by increasing the ability of these cells to endure free radical exposure and impairment in substrate metabolism. It is noteworthy that when mouse pancreatic islets are exposed to IL-1$\beta$ there are similar NO generation as observed in rat pancreatic islets [30]. Moreover, aconitase activity is also inhibited by IL-1$\beta$ in mouse islets, but there is no significant impairment in glucose metabolism or ATP generation [31,32]. These observations suggest that mouse pancreatic islets are able to better endure a partial impediment of the Krebs cycle than rat islets. Apparently SAM improves this particular susceptibility of rat $\beta$-cells to inhibition of the Krebs cycle.

As mentioned above, prolonged islet exposure to IL-1$\beta$ and NO affects ATP generation by blocking aconitase [17]. It is conceivable that NO also induces other deleterious effects on $\beta$-cells, like impairment in the mitochondrial electron chain transport, inhibition of DNA synthesis and ADP-ribosylation of enzymes of the glycolytic pathway [33]. However, observations that the cytokine does not impair the glycolytic pathway [34] nor the conversion of pyruvate into the acetyl-coenzyme-A residues [35] emphasizes the importance of the inhibition at the Krebs cycle level. Interestingly, not all steps of the cycle are inhibited to the same extent by IL-1. Thus, while the cytokine impairs both glucose and leucine metabolism, it does not decrease the oxidation of glutamine [35]. Glutamine enters the Krebs cycle as ketoglutarate, in a step distal to the enzyme aconitase. If one considers that SAM gains access to the Krebs cycle mostly as succinate [12], also distal to aconitase, it is conceivable that the ester may increase $FADH_2$ and NADH generation and consequent ATP production by by-passing the decreased aconitase activity. Moreover, not all electrons from oxi-

dized substrates enter the electron chain transport at the $NAD^+$ level, and electrons from succinate may pass from FAD directly to ubiquinone [36]. This can also contribute to an improvement in cell function in the context of IL-1$\beta$-induced decreased NADH production. It must be emphasized that the protection afforded by SAM is partial, and that the ester does not improve islet function when added for a short period (1 h) following 24 h exposure to IL-1$\beta$. This suggests that there are some deleterious effects induced by NO that can not be prevented by SAM and/or that the ester can not revert a situation characterized by severe cell damage, as observed following 24 h exposure to IL-1$\beta$.

Up to now most attempts to prevent the outbreak of IDDM have been directed towards suppression of the autoimmune assault [3]. Recent data suggest that in this context attention must also be paid to repair mechanisms triggered by the $\beta$-cells (reviewed in [2]). It may well be that SAM, by providing a substrate that can be effectively oxidized by damaged $\beta$-cells, is providing the energy that will allow a more efficient activation of these repair mechanisms.

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