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Insight statement

Type-2 diabetes mellitus (T2DM) is a tremendous health problem worldwide. Our work demonstrates that the microelectrode array (MEA) technique is an excellent tool to study *in vitro* the molecular basis of functional changes in beta-cells Occurring in the development of type 2 diabetes mellitus. First, we established an *in vitro* model that allow long-term investigations of beta-cell function by registration of electrical activity from isolated islets with the MEA technique. In a second step we applied oxidative stress to the islets which is crucial in the development of T2DM and evaluated appropriate protection mechanisms. This is fundamental work that enables monitoring electrical activity of murine islets in controlled *ex vivo* situations during long-term exposure to defined diabetes-associated, e.g. oxidative stress, glucolipotoxicity, and diabetes-inducing agents.

Sentence of 20 words

Extracellular recordings of electrical activity from pancreatic islets permit long-term measurements of beta-cell function and reveal oxidant-induced damage and rescue.

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Long-term culture and functionality of pancreatic islets monitored by microelectrode array

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Key words: microelectrode array (MEA), beta-cell, oscillations, electrical activity, insulin secretion, oxidative stress, hydrogen peroxide, superoxide dismutase (SOD), SOD mimetic, long-term culture, human islet

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Abstract

Extracellular recordings of glucose-induced electrical activity from mouse islets of Langerhans on microelectrode arrays (MEA) are an innovative and powerful tool to address beta-cell (patho-)physiology. In a dual approach we tested whether this technique can detect concentration-dependent drug effects as well as characterize alterations in beta-cell activity during prolonged culture. First we established conditions that allow long-term investigation of beta-cell function by recording electrical activity. The results provide the first measurements of beta-cell membrane potential oscillations of individual murine islets during long-term culture. Oscillations were recorded for up to 34 days after islet isolation. Importantly, the glucose dependence of electrical activity did not change over a period of one month. Thus we can follow electrophysiological changes of individual islets induced by alterations in the beta-cell environment over weeks. Second, we used the MEA technique to assay beta-cell damage induced by oxidative stress and to evaluate appropriate protection mechanisms. Oxidative stress plays a key role in the development of type 2 diabetes mellitus (T2DM). Examination of the acute effects of H_2O_2 on electrical activity showed the oxidant concentration-dependently reduced electrical activity. The superoxide dismutase mimetic, tempol, protected against the detrimental effects of H_2O_2. In conclusion, we demonstrated that MEA recordings can be used to address disease-related mechanisms and protective interventions in beta-cells. In the future, this fundamental work should enable the monitoring of the electrical activity of islets of Langerhans under controlled *ex vivo* conditions including long-term exposure to oxidative stress, glucolipotoxicity, and other diabetes-inducing agents.

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Introduction

Insulin secretion of pancreatic beta-cells is determined by the degree of electrical activity, i.e. phases of depolarized membrane potential with action potentials and repolarized interbursts. Recently, we succeeded in recording electrical activity of isolated mouse islets of Langerhans with planar, extracellular electrodes arranged as a microelectrode array (MEA) [1]. This approach was confirmed by Raoux and coworkers [2]. The MEA system allows quantifying electrical activity of beta-cells by calculating the fraction of plateau phase (FOPP, percentage of time with spike activity). We demonstrated that the glucose dependence of FOPP measured with MEA equals that measured with sharp intracellular microelectrodes and is almost identical with the glucose dependence of insulin secretion measured with isolated mouse islets [3]. In addition, the MEA technology enables detection of the first phase of electrical activity elicited by a rise in glucose from a sub-stimulatory to a stimulatory concentration and allows simultaneous measurement of fluctuations of electrical activity and cytosolic Ca^{2+} concentration [1].

These results convincingly demonstrate that MEAs are an excellent and unique tool for reliable measurements of FOPP. MEAs are superior to conventional electrophysiological methods since they are 1) non-invasive, 2) use whole islets that – in contrast to single cells – consistently display oscillations of electrical activity, and 3) are easy to handle, thus increasing the experimental through-put enormously.

In humans, T2DM develops over many years. Current *in vitro* models to investigate alterations in the oscillatory activity of pancreatic islets seldom exceed a time period of several hours and are thus inadequate to study long-term changes. We demonstrate, for the first time, that the MEA approach allows monitoring of electrical activity and glucose-responsiveness of cultivated islets over a period of weeks. Increased oxidative stress within beta-cells due to excessive fuel intake or increased hormone levels (e.g. angiotensin II, endothelin) is a key event in the development of T2DM [4]. Since antioxidant defence mechanisms are low in beta-cells the characterization of strategies to prevent oxidative stress is a promising approach to identify beta-cell protective drugs. It is well known that oxidative stress reduces or even completely prevents glucose-mediated alterations in electrical activity [5], thus the ability to make extended electrical measurements is attractive. We show the MEA technology is very sensitive, allows detection of small changes in electrical activity

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caused by acute treatment of islets with an oxidant and can be used to determine the protective effect of a drug mimicking superoxide dismutase.

Taken together, we demonstrate the suitability of the MEA-based approach 1) to develop *in vitro* models for the study of long-term changes in islet function, 2) to investigate effects of diabetes-promoting conditions, i.e. oxidative stress, and 3) to test strategies for protection of islets against diabetes-associated functional cell damage.

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Research Design and Methods

Islet preparation

Experiments were performed using intact islets of Langerhans isolated from adult C57Bl/6N mice (Janvier, France). The principles of laboratory animal care were followed according to German laws. Mice were euthanized by CO_2. Islets were isolated by collagenase digestion of the pancreas and cultured up to 34 days in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

Solution and chemicals

Measurements of extracellular membrane potential were performed at 37°C in a solution containing in mM: 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES, pH 7.4, and glucose as indicated. The incubation medium for determining insulin secretion contained (in mM): 122 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.1 $MgCl_2$, 10 HEPES, pH 7.4. RPMI 1640, fetal calf serum, and penicillin/streptomycin were provided by Invitrogen (Karlsruhe, Germany). All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany).

Recording setup for measuring membrane potential by extracellular electrodes

Extracellular membrane potential recordings were obtained with the MEA technique [6] using a MEA 1060-inv-standard amplifier system with software MC-Rack (Multi Channel Systems (MCS), Reutlingen, Germany). Data were low-pass filtered at 25 Hz and sampled at 1 kHz. Titanium-nitride electrodes had a diameter of 30 µm (200/30-Ti; MCS). In experiments with acute application of drugs one islet was placed on one of the 64 electrodes by means of a glass holding pipette with a tip angle of 30° (Reproline, Rheinbach, Germany) and a micromanipulator (Eppendorf, Hamburg, Germany). Extracellular voltage changes were only recorded from the electrode where the islet was placed using the grounded bath electrode as reference [1]. In long-term culture experiments several islets were placed on different electrodes within the array.

Experimental procedure for measuring extracellular membrane potential

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For acute drug application islets were used after 1–3 days in culture. Islets were incubated in 3 mM glucose for 10 to 20 min prior to experiments. Single islets were then transferred to the MEA bath chamber using a holding pipette and fixed to an electrode with gentle mechanical pressure. The islet was continuously perifused with bath solution throughout the experiment. Recordings always started in 3 mM glucose which gave a base-line with no electrical activity. Oscillatory activity was usually followed for 30 – 45 min for each condition and evaluated at periods of steady-state oscillations for the last 5-10 min before a new manoeuvre started. For long-term culture experiments the islets were kept in culture medium (RPMI, 11.1 mM glucose) on the MEA for the indicated time period. The culture medium was changed every second day. Each experiment started in culture medium before changing to the bath solution. For quantification of electrical activity the fraction of plateau phase (FOPP) was determined by dividing a distinct time interval by the time with bursting activity within this distinct time interval.

Measurement of insulin secretion

After preparation islets were kept overnight in medium with 11.1 mM glucose. To determine insulin secretion, batches of 5 islets were incubated for 60 min at 37°C with the indicated substances. Insulin was determined by radioimmunoassay using rat insulin (Crystal Chem. Inc., USA) as the standard.

Presentation of results

The recordings are representative of results from different islets; islets from at least 3 different mice were used for each series of experiments. Values are given as means ± SEM for the indicated number of experiments. Statistical significance was assessed by ANOVA followed by the Student-Newman-Keuls post-test. A value of $p \leq 0.05$ was considered to be significant.

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Results and Discussion

Long-term measurements of beta-cell electrical activity

To study the effects of a diabetes-promoting environment, e.g. ROS, on the electrical activity of mouse beta-cells in more detail, it would be of great advantage to record changes over several days. However, all current electrophysiological techniques used in beta-cell research provide only short-term measurements of electrical activity, i.e. a range of minutes, or in exceptional cases, hours. In experiments with conventional microelectrodes or patch-clamp experiments the membrane potential measurement is regularly disturbed by leakage currents. Moreover, it is not possible to study electrical activity of the same islet on different days. Therefore, we developed a system allowing long-term culture of isolated islets and, most importantly, regular measurements of electrical activity of individual islets during culture. We validated physiological dynamics, i.e. the glucose responsiveness of the islets cultured on the MEA in the presence of 11.1 mM glucose and the appearance of oscillations over several weeks. As shown in Fig. 1A islets maintain their ability to respond adequately to glucose with oscillations up to 34 days. It was possible to record independent electrical activity simultaneously from several islets in contact with different electrodes on the MEA. Thus, the rate of successful experiments was increased compared to conventional electrophysiological methods.

As observed in acute experiments, in 3 mM glucose no oscillations were detected even after 34 days in culture. Differences in the oscillations induced by 10 or 15 mM glucose, respectively, could be distinguished over time in culture showing that the glucose dependence of electrical activity was preserved over several weeks (Fig.1B). The data indicate that glucose metabolism is intact after long-term culture. FOPP at 10 and 15 mM glucose, respectively, did not change over time. To evaluate possible culture time-dependent changes in electrical activity, FOPP was summarized for experiments measured in the first week of culture (day 6 and 7) and compared with experiments performed in the last culture week (day 33 and 34). FOPP amounted to 32±3 % (n=8) and 36±2 % (n=7) at 10 mM glucose and to 70±6 % (n=8) and 72±5 % (n=7) in 15 mM glucose at day 6/7 and day 33/34, respectively. The data clearly demonstrate that FOPP at 10 and 15 mM glucose, respectively, did not change over time in culture. Notably, the number of non-responding islets is negligible with the MEA technology in contrast to microelectrode impaling. This paves the way to

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perform long-term experiments of electrical activity with low concentrations of redox-active compounds or diabetes-inducing agents and to study progressive changes evoked by gluco-, lipo- or glucolipotoxicity. Raoux and coworkers reported reduced action potential frequency of clonal INS-1E cells kept on a MEA after 3 days in elevated glucose concentration [2]. Moreover, the method permits co-culture of islets with other tissues to investigate their influence(s) on electrical activity.

In summary, the data presented show that MEA technology offers numerous novel and interesting long-term applications in beta-cell research, e.g. investigation of chronic effects of diabetes-inducing environments on electrical activity or the development of an *in vitro* model for testing short and long-term effects of potential antidiabetic drugs.

MEA technology detects protection against oxidative stress-induced pathophysiological changes of beta-cell electrical activity

Oxidative stress is an important pathogenic factor that contributes to the development of T2DM [4a]. We have shown that up-regulation of the antioxidant enzymes catalase (Cat), glutathione peroxidase (Gpx) and superoxide dismutase (SOD) in primary mouse beta-cells protects against apoptosis and loss of beta-cell function, respectively, that was induced by oxidative stress [5a] This is in agreement with observations of Lortz and co-workers who showed that overexpression of antioxidant enzymes reduces cytokine-induced cytotoxicity in insulin-secreting RINm5F cells [7]. H_2O_2 is one of the reactive oxygen species markedly contributing to oxidative stress in pancreatic islets. It is known to interact with several parameters of beta-cell stimulus-secretion coupling [8]. In the present study we investigated H_2O_2-evoked changes in electrical activity of mouse beta-cells and tested whether the membrane-permeable SOD mimetic, tempol [9], protects beta-cells against H_2O_2-induced insult. First, we applied different concentrations of H_2O_2 in the presence of 10 mM glucose resulting in a concentration-dependent inhibition of FOPP that was well resolved by the MEA technology (Fig. 2A, B). Second, islets pre-incubated in 1 mM tempol prior to the addition of 300 µM H_2O_2 were compared to those exposed solely to 300 µM H_2O_2. To avoid direct interaction of tempol and H_2O_2, tempol was removed immediately before addition of H_2O_2. H_2O_2 significantly reduced the FOPP in the presence of 10 mM glucose but was without effect after 30 min of pre-incubation with 1 mM tempol (Fig. 2C, D). The SOD mimetic exerted a protective effect against the

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reduction of insulin secretion by 100 µM H_2O_2.In experiments without pre-treatment insulin secretion was reduced, but was unchanged after 15 min pre-incubation with the SOD mimetic (Fig. 2E). Tempol alone did not influence insulin secretion (2.91±0.36 ng insulin/(islet*h) in the presence of 15 mM glucose *vs* 3.18±0.29 ng insulin/(islet*h) in the presence of 15 mM glucose and tempol; n=4; n.s.; not shown). The different efficacy of H_2O_2 on insulin secretion and electrical activity is most likely due to differences in diffusion in the two measurement systems (1 h steady-state incubation for insulin secretion *vs* perifusion for measurement of electrical activity). The results clearly demonstrate that tempol preserves insulin secretion by circumventing the detrimental effect of H_2O_2 on stimulus-secretion-coupling. This agrees with our previous observations that up-regulation of antioxidant enzymes protects beta-cells against oxidative stress [5a]. The new data also show that the SOD plays a crucial role in the antioxidant defence of beta-cells and seems to be more important than Cat and Gpx since an SOD mimetic alone is sufficient to protect against an oxidative insult. At first glance it seems astonishing that an SOD mimetic protects electrical activity and insulin secretion against H_2O_2 since SOD does not degrade H_2O_2. It is assumed that tempol-dependent protection of the islets is due to detoxification of H_2O_2-induced ROS production including O_2^-. First, H_2O_2 leads to a partial depolarization of the mitochondria [5b] and a decrease of the mitochondrial membrane potential is assumed to increase ROS production. Second, ROS, including H_2O_2, can induce mitochondrial ROS release [10]. This mechanism has been extensively studied in cardiomyocytes and involves the mitochondrial permeability transition pore and/or the anion channel of the inner mitochondrial membrane. In both cases formation of superoxide anions in the mitochondria would rise and potentiate ROS generation and action. We suggest that this vicious circle is stopped by the SOD mimetic.

We have shown that a SOD mimetic protects beta-cells against acute oxidative stress pointing to the central role of SOD in antioxidant defence of pancreatic islets. Our experiments demonstrate that glucose-responsiveness of isolated islets cultured on MEAs is stable for up to 34 days. These data indicate that MEA technology is a valuable tool to investigate long-term effects of drugs influencing beta-cells via membrane potential-dependent pathways. The results suggest the feasibility of developing MEA-based *in vitro* disease models where islets are chronically

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challenged by glucolipotoxicity or stress-inducing agents thus enabling evaluation of intervention strategies.

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Figure Legends:

Figure 1: Electrical activity of isolated mouse islets in long-term culture in the presence of 11.1. mM glucose. A) Typical recordings showing electrical activity induced by 10 and 15 mM glucose and lack of activity in bath solution with 3 mM glucose after 6 and 34 days in culture. B) Quantification of the FOPP in the presence of 10 and 15 mM glucose for different days of culture. *P≤0.05, **P≤0.01, ***P≤0.001. Statistical differences between the FOPP in 10 and 15 mM glucose at each culture day. The n-values within the columns indicate the number of islets. 5 different preparations are included in this series of experiments.

Figure 2: Effect of H_2O_2 and the SOD mimetic tempol on insulin secretion and electrical activity of mouse beta-cells. A) Concentration-response curve of the effect of H_2O_2 on the fraction of plateau phase (FOPP). n=3-14. **P≤0.01, ***P≤0.001 compared to control without H_2O_2. B) One typical recording out of 14 showing the application of 0,3 mM H_2O_2 in the presence of 10 mM glucose on electrical activity. C) Original recordings in bath solution with 10 mM glucose (upper trace), 10 mM glucose and 0.3 mM H_2O_2 (middle trace) as well as in the presence of 10 mM glucose and 0.3 mM H_2O_2 after preincubation with 1 mM tempol (lower trace). D) Quantification of the results presented as FOPP. E) Insulin secretion of islets *in vitro* in the presence of 15 mM glucose, + 0.1 mM H_2O_2, + additional preincubation with 1 mM tempol.

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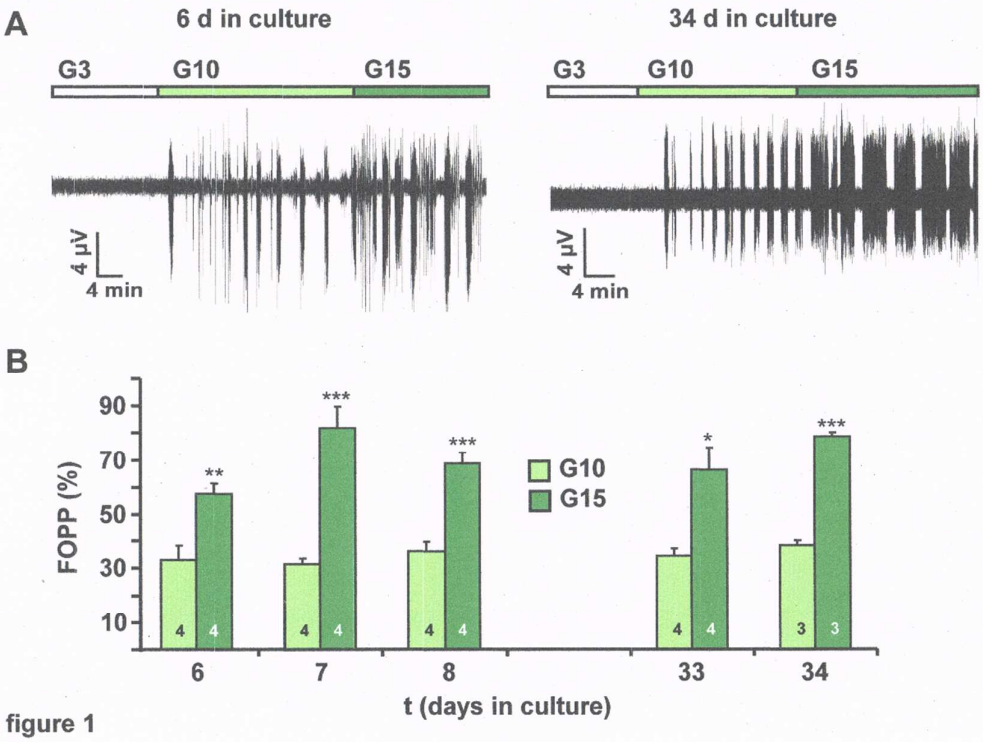

figure 1

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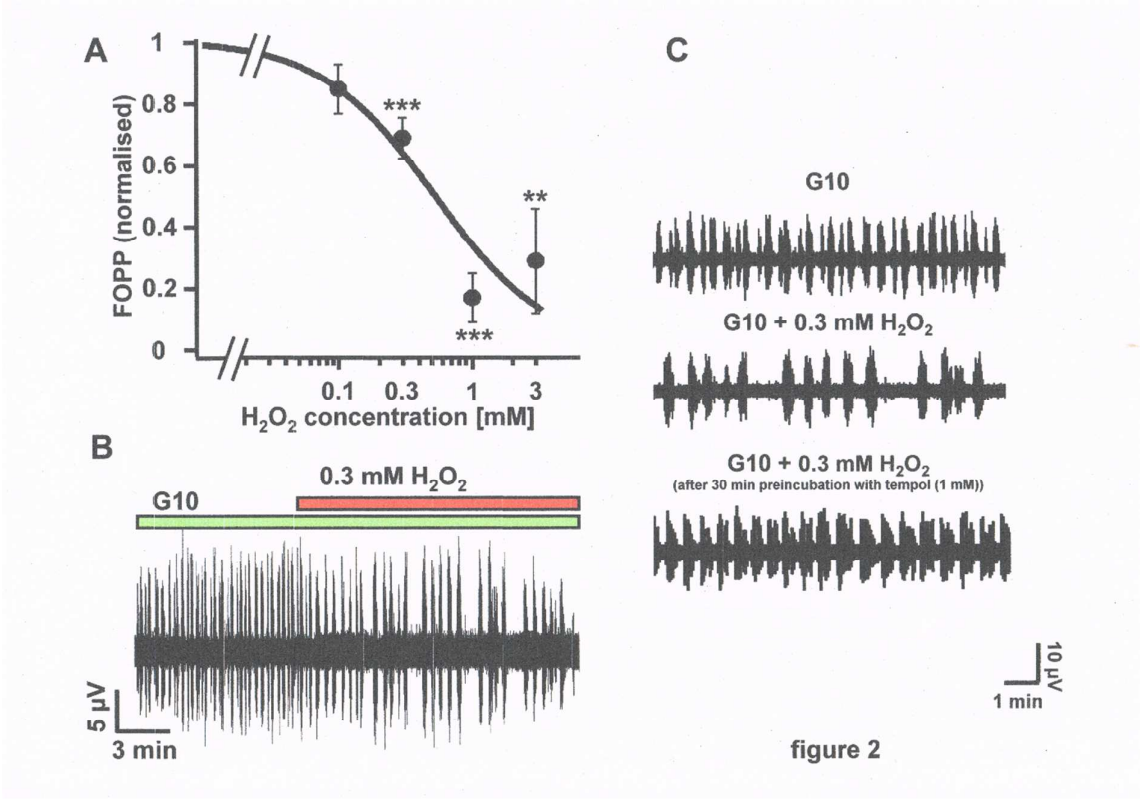

figure 2

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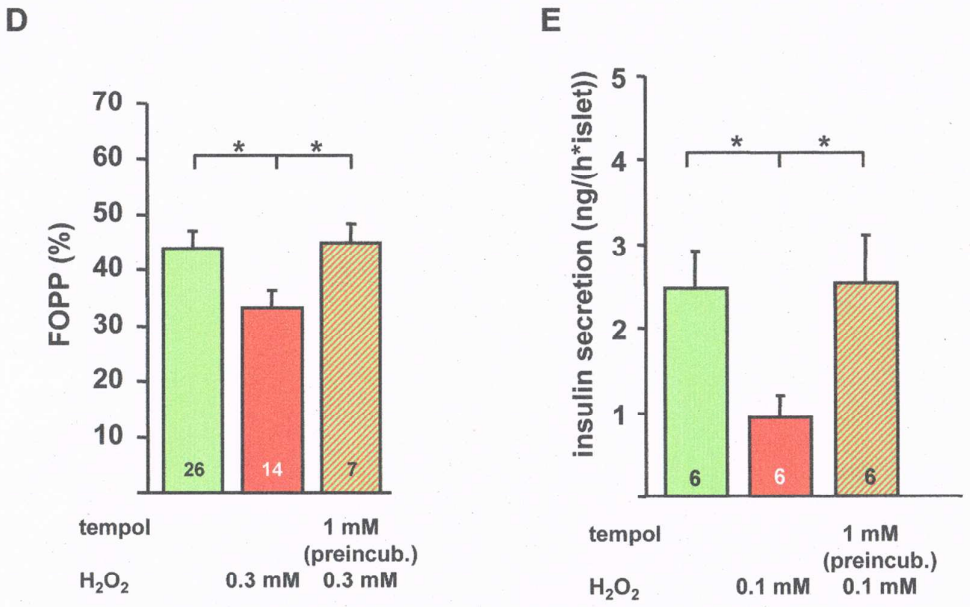

figure 2

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