Reoxygenation Injury Affects Isolated Islet Response to Fatty Acid Stimulation

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Hyperlipidemia is frequently associated with hyperinsulinemia, but because the effects of fatty acids on insulin secretion in in vitro studies using isolated perifused islets have mostly been described with supraphysiological concentrations of fatty acids, it has remained uncertain whether elevated lipid levels contribute to hyperinsulinemia by their direct stimulation of insulin secretion. In the present study, we have identified reoxygenation injury in isolated islet function as a contributing factor in the failure of physiological concentrations of free fatty acids to stimulate insulin secretion in isolated perifused islets. Reoxygenation of isolated islets is associated with the production of reactive oxygen species, which impair islet function. We have found that pretreatment of freshly isolated islets with the antioxidant glutathione (GSH), as well as a 24-hour preculture of isolated islets under appropriate conditions, enhanced their sensitivity to fatty acid stimulation.

IT HAS LONG BEEN ESTABLISHED that hyperlipidemia is frequently associated with impaired glucose regulation,[1,2] which presents as insulin resistance characterized by hyperglycemia and hyperinsulinemia.[3] More recently, it has been suggested that fatty acids play a role in the development of insulin resistance,[4,5] which has been proposed as a primary defect in patients with type 2 diabetes.[6-8] However, the mechanism by which fatty acids induce hyperglycemia and hyperinsulinemia remains unclear. It is generally believed that fatty acid oxidation generates metabolites, which inhibit glucose oxidation in peripheral tissues, such as the skeletal muscle, while at the same time enhancing gluconeogenesis in the liver.[1,3-5] The ensuing hyperglycemia is then thought to provoke excessive insulin secretion, resulting in the coexistence of hyperglycemia-with hyperinsulinemia.[3,5] The problem with this dogma is that it fails to consider the fact that fatty acid–induced inhibition of glucose oxidation also occurs in pancreatic islets,[9] and since glucose stimulation of insulin secretion is dependent on glucose oxidation,[10,11] the expected effect of glucose on insulin secretion would not occur easily. Therefore, alternative hypotheses on the relationship between elevated fatty acid levels and hyperinsulinemia need to be considered.

Studies have shown that fatty acid oxidation stimulates insulin secretion, while inhibiting glucose-stimulated insulin secretion,[12-15] raising the possibility that the direct effect of fatty acids on insulin secretion by pancreatic islets may, at least in part, contribute to the development of hyperinsulinemia by hyperlipidemia. The major drawback with this hypothesis is that either supraphysiological concentrations of fatty acids were used or fatty acid effects were only observed in the presence of high glucose concentrations in most of the previous studies,[13-19] which raised doubts about the direct effects of fatty acids on insulin secretion.

It has been shown that reoxygenation injury generates reactive oxygen species,[20] which interfere with glucose oxidation[21] and consequently inhibit glucose-stimulated insulin secretion. In recent experiments, we have observed that pretreatment of freshly isolated perifused murine islets with the antioxidant, glutathione (GSH), enhanced their response to stimulation with glucose.[22] Other investigators have also shown that the treatment of cryopreserved isolated human islets with the antioxidant, butylated hydroxyanisole (BHA), improved glucose-induced insulin release in the islets.[23] These observations suggest that the generation of oxygen free radicals in isolated perifused islets may have an adverse effect on their response to secretagogues. The purpose of the present study was to test the hypothesis that reoxygenation injury may be responsible for the inability of physiological fatty acid concentrations to stimulate insulin secretion in freshly isolated perifused islets. We have, therefore, tested the effects of low concentrations of free fatty acids on islets, after either pretreatment with GSH or following a 24-hour culture at 37°C in an atmosphere of humidified 95% air and 5% CO_2.

MATERIALS AND METHODS

Adult female CD-1 albino mice were obtained from Charles River (Raleigh, NC). Mice were used in the present study because our previous studies, in which low concentrations of fatty acids had no effect on insulin secretion, were performed with murine islets.[14-16] The mice were housed in the Duke University vivarium in stainless steel cages and maintained at a temperature of approximately 37°C with a 12:12-hour light/dark cycle. They were placed on a diet consisting of laboratory chow diet (Purina, St Louis, MO). The procurement and method of use of the animals in the study were approved by the Duke University Medical Center review board for the welfare of animals. Highly purified bovine serum albumin (BSA; fraction V) free of fatty acids and insulin-like activity, reduced GSH, fatty acids, glucose, and all chemicals for buffer solutions were purchased from Sigma Chemical (St Louis, MO). Monoiodinated ^{125}I-insulin was obtained from New England Nuclear (Boston, MA).

Preparation of Fatty Acid Perifusates

The unsaturated fatty acid linoleate ($C_{18:2}$) in its oily form, as obtained from Sigma, was readily solubilized in the perifusate buffer supplemented with 1% albumin. Palmitate ($C_{16:0}$), a saturated fatty acid purchased in its solid form, was first dissolved in methanol before emulsification in the albumin-supplemented perifusate buffer. The final concentration of methanol was 0.05%, but the emulsion was heated to remove the methanol which, in trace amounts, had no effect on insulin secretion.[16] The perifusate buffer was a modified Krebs-Ringer bicarbonate (KRB) solution consisting of 120 mmol/L NaCl, 5 mmol/L KCL, 1.1

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mmol/L $MgCl_2$, 2.5 mmol/L $CaCl_2$, 25 mmol/L $NaHCO_3$, 5.5 mmol/L glucose, 1% albumin, with or without 10 mmol/L GSH. The KRB solution was maintained at pH 7.4 by continuous gassing with a mixture of 95% O_2/5% CO_2.

Isolation of Islets

On each day of the experiment, two mice were fasted for at least 4 hours, but were allowed free access to water until they were killed. Islets were isolated by a modification of the original method described by Lernmark.[24] The pancreata were removed and minced and the minced tissue was then added to 8 mL Hanks (maintained at 37°C), followed by the addition of 2 mL Hanks containing 15 mg collagenase kept on ice for 30 minutes before use. The mixture was shaken vigorously in a 37°C water bath for 12 minutes. The digested contents were filtered through a double layer of gauze to remove undigested pancreatic tissue and centrifuged at 450 rpm for 2 minutes at 4°C. After decanting the supernatant, the pellet was washed four times by resuspending and dispersing the pellet each time in fresh Hanks, followed by centrifugation and aspiration of the supernatant. The final pellet was dispersed in Hanks solution and poured into a dish from which islets were selected by handpicking with a pipette under a dissecting microscope. Six islets were either placed directly into miniature plastic flow-through perifusion chambers for immediate experiments with control (untreated) or GSH-treated islets or after culture for 24 hours.

Cell Culture

Islets, which were cultured before use, were isolated under sterile, aseptic conditions. The islets were then placed in sterile culture dishes containing RPMI 1640, supplemented with 10% fetal calf serum and 1% mixture of antibiotics (penicillin/streptomycin). The culture dishes were incubated for 24 hours at 37°C in an atmosphere of humidified 95% air and 5% CO_2. Following the 24-hour culture, intact islets were handpicked for experiments using a pipette under a dissecting microscope.

Perifusion of Islets

In each experiment, six islets were placed into a miniature flow-through perifusion chamber and preperifused at the rate of 1 mL/min at 37°C for 1 hour. In experiments with freshly isolated islets, the islets were preperifused in the absence (control) or presence of 10 mmol/L GSH. In the experiments with 24-hour cultured islets, GSH was excluded from the preperifusion. The perifusate buffer was maintained at pH 7.4 by continuous gassing, and following the preperifusion in the presence or absence of GSH, basal samples were taken on ice for 20 minutes before the perifusion was continued with the addition of a given fatty acid to the perifusate buffer without GSH. Solutions were changed using a stopcock, and effluent perifusate samples were collected on ice at 5-minute intervals and stored frozen until radioimmunoassay for insulin.[25]

Data Analysis

Data represent the mean ± SE. Statistical evaluation was performed using an ANOVA computer program (GraphPad Software, San Diego, CA), and depending on the outcome of ANOVA, the Bonferroni correction[26] was used to assess the significance of differences between the means. In the case of determining the significance of the difference between the means of only two samples, Student's *t* test was used. In all cases, a value of $P < .05$ was considered significant.

RESULTS

Effect of Linoleic Acid on Insulin Secretion in Control and GSH-Treated Islets

In previous studies, although high concentrations of linoleic acid ($C_{18:2}$) had a rapid effect on insulin secretion, concentrations of this fatty acid less than 1 mmol/L had no appreciable effect in freshly isolated islets during 20 minutes of perifusion.[15,16] Consistent with those observations, in the present study, 0.25 mmol/L linoleic acid had no effect on insulin secretion above basal rate in control untreated freshly isolated perifused islets (Fig 1). However, as apparent in Fig 1 and in Table 1, the basal rate of insulin secretion in the untreated islets was significantly higher than that of GSH-treated islets ($P < .001$). Following pretreatment of islets with GSH, insulin secretion increased significantly above basal ($P < .05$) when 0.25 mmol/L of linoleate was added to the perifusate without GSH (Fig 1). The effect of this fatty acid on insulin secretion in GSH-treated islets was dose-related, as seen in Fig 2, which shows a sustained increased rate of insulin secretion in the presence of 0.75 mmol/L of the fatty acid.

Effect of 0.75 mmol/L Linoleate or Palmitate on Insulin Secretion in Cultured Islets

Since reperfusion injury appears to occur immediately after reoxygenation of isolated islets,[27] and since 0.75 mmol/L linoleate caused a sustained stimulation of insulin secretion (Fig 2), the effect of 0.75 mmol/L of the polyunsaturated fatty acid, linoleate ($C_{18:2}$), or the saturated fatty acid, palmitate ($C_{16:0}$), was tested in intact islets after 24 hours of culture. In previous studies with freshly isolated perifused islets, concentrations of palmitate up to 10 mmol/L had no stimulatory effect on insulin secretion.[16] However, as apparent in Fig 3, 0.75 mmol/L of either palmitate or linoleate caused a significant stimulation ($P < .05$) of insulin secretion that appeared to occur in a biphasic manner in the cultured islets, in contrast to the GSH-treated freshly perifused islets (Figs 1 and 2). Yet, as apparent in Table 1, the basal rate of insulin secretion was similar in GSH-treated freshly perifused islets and in the 24-hour cultured islets. Also, the basal rate of insulin secretion

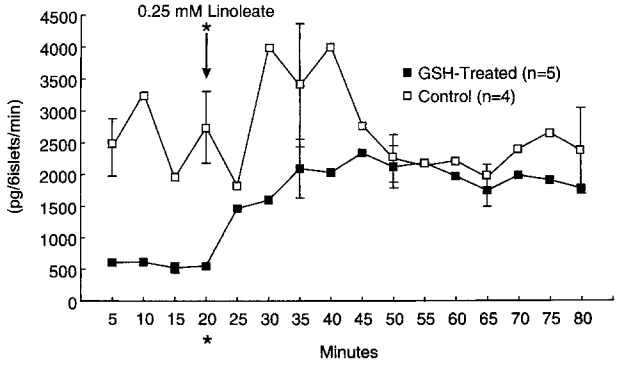

Fig 1. Effect of 0.25 mmol/L linoleic acid on insulin secretion. Isolated islets were preperifused in the absence (control) or presence of 10 mmol/L GSH for 1 hour, in a buffer containing 2 mmol/L glucose and 1% albumin. After collection of basal effluent samples for 20 minutes, 0.25 mmol/L linoleate was added and samples were also collected for insulin assay.

Table 1. Basal Rates of Insulin Secretion in the Three Groups of Islets

Variable	Control	GSH-Treated	24-Hour Cultured Islets
Basal rate	2,600 ± 275	570 ± 20*	565 ± 25*
No. of experiments	4	5	4

NOTE. The values, which represent the mean ± SEM in pg/6 islets/min, were statistically evaluated by ANOVA.
*$P < .001$ (*v* control).

in these two groups was significantly lower ($P < .001$) than that observed in control fresh islets perifused without GSH-treatment (Table 1). Interestingly, although the perifusion with each fatty acid was started at the same time point, there was a notable difference in the time course of the effects of the two fatty acids on insulin secretion in the cultured islets perifused without GSH treatment (Fig 3).

DISCUSSION

The present study suggests that reoxygenation injury may, at least partially, explain the inability of low physiological concentrations of fatty acids to enhance insulin secretion in freshly isolated perifused islets seen in previous studies.[12-16] It is noteworthy that although physiological concentrations of palmitate had failed to stimulate insulin secretion under basal conditions, palmitate potentiated the release of insulin induced by high glucose concentrations through mechanisms that appear to involve both an increase in intracellular Ca^{2+} concentrations and the provision of lipid mediators.[28,29] Since high concentrations of fatty acids were able to stimulate insulin secretion in previous studies,[13-18] our present observations would be consistent with a hypothesis that the generation of free radicals during reoxygenation following a period of hypoxia affects the sensitivity of freshly isolated islets to fatty acid stimulation. The findings in the present study are consistent with those from other studies, which showed that the isolated islet response to stimulation with glucose can be enhanced by pretreatment with the antioxidant GSH.[22,30] It has also been reported that exogenous GSH supplementation enhances glucose-stimulated insulin secretion in elderly individuals with impaired glucose tolerance.[31]

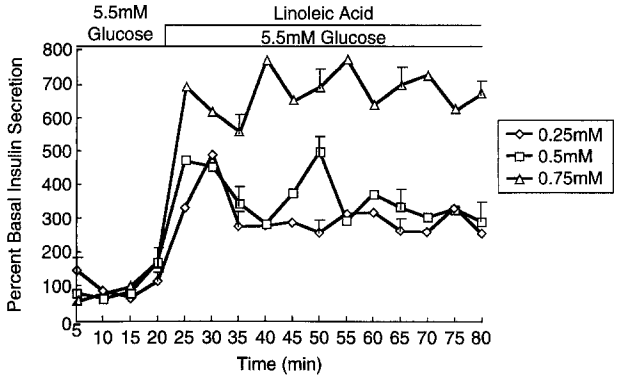

Fig 2. Dose-response effect of linoleic acid on insulin secretion. Following preperifusion of islets with the GSH-containing buffer and collection of basal samples, a given concentration of the fatty acid was added to the perifusate without GSH, in separate experiments, each of which was performed at least 4 times. Insulin content in the samples collected during the fatty acid perifusion was assessed as the percent of that of the basal samples from each experiment.

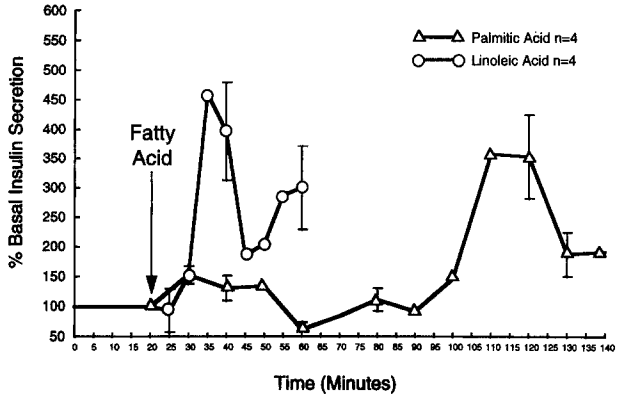

Fig 3. Effects of palmitate and linoleic acid on cultured islets. Isolated islets were cultured at 37°C for 24 hours in RPMI 1640 medium containing 10% fetal calf serum and a mixture of antibiotics, in a humidified atmosphere of 95% air and 5% CO_2. Intact islets were then selected for experiments in which the islets were preperifused in the absence of GSH. After preperifusion and collection of basal samples, 0.75 mmol/L of either palmitate or linoleate was added to the perifusate. Insulin output in the presence of the fatty acids was assessed as the percent of basal output in each experiment.

There is substantial evidence that link reoxygenation injury with overproduction of oxygen free radicals or reactive oxygen species.[20,32] It is well established that although all of the major antioxidant enzymes are present in pancreatic islets, they occur in low amounts,[33] and this antioxidant deficiency could be exacerbated by losses during the isolation process. Consequently, isolated islet function may be impaired unless corrected by the detoxification of these reactive oxygen species with antioxidant supplements, as is apparent in the present and other studies.[22,23,30] It is noteworthy that transplantation of isolated islets is also associated with reoxygenation injury,[27] which has been attributed to reactive oxygen species, such as nitric oxide (NO), which requires GSH for detoxification and restoration of cellular function.[34,35]

It has been shown that posthypoxia/reoxygenation injury occurred rapidly in isolated islets, resulting in cell death.[27] This observation is consistent with the fact that when islets are placed in culture, there is a decrease in the number of surviving intact islets. Presumably, these intact islets are no longer vulnerable to damage after surviving in culture in a controlled reoxygenated atmosphere. Therefore, the cultured islets may become more sensitive to stimulation with secretagogues. This probably would explain why we found that, following a 24-hour period of culture, isolated islets were stimulated to release insulin by physiological concentrations of fatty acids. Our data show that freshly isolated untreated islets are hypersecretory compared with GSH-treated islets, and these data are consistent with those from another study, which showed that pretreatment of isolated perifused islets with the antioxidant, butylated hydroxyanisole (BHA), reduced the basal rate of insulin secretion while enhancing glucose-stimulated insulin secretion.[23] Indeed, it has been shown that reactive oxygen species activate signal transduction in biological processes.[36] The fact that NO, an oxygen free radical, causes cellular dysfunction,[34]

including the activation of insulin secretion,[37] but can be deactivated by GSH,[35] also supports our finding of a reduced rate of basal insulin secretion in the GSH-treated islets. Indeed, it has been found that when normal islets were cultured in the presence of high concentrations of fatty acids, NO production and basal insulin secretion were increased.[38] In the present study, we found that, following 24-hour culture of islets in RPMI 1640 medium containing no fatty acid, the basal rate of insulin secretion was reduced compared with freshly isolated non–GSH-treated islets. The similarity in the reduced basal rate of insulin secretion seen in both the 24-hour cultured islets and the fresh GSH-treated islets appears to suggest a restoration of normal isolated islet function that permits sensitivity to physiological fatty acid concentrations in the two groups of islets. However, it is unclear why GSH-treated islets responded to fatty acid stimulation without a biphasic insulin secretion, as was seen in the cultured islets. As was noted in the present study, freshly isolated perifused islets appear to be normally hypersecretory. It is possible that the acute suppression of the high secretory rate by GSH treatment may have induced some kind of sustained compensatory release of insulin in response to stimulation, thus preventing the biphasic secretion.

Previous studies had shown that fatty acid stimulation of insulin secretion depended on fatty acid oxidation by pancreatic islets.[9,12-14] It is, therefore, of interest to note that, in the present study, the response of cultured islets to stimulation with fatty acids was related to the structural characteristics of a given fatty acid, consistent with a previous report.[15] Hence, we found in this study that palmitic acid ($C_{16:0}$) stimulation of insulin secretion occurred later than that of linoleic acid ($C_{18:2}$), presumably because of the faster rate of uptake and oxidation of linoleate compared with that of palmitate.[39,40] In summary, in agreement with earlier studies,[22,23,30] we have identified pretreatment of isolated islets with an antioxidant, as well as a 24-hour preculture of islets under appropriate conditions, as effective means of minimizing the deleterious effects of reoxygenation injury on isolated islet function. When either of these two approaches are taken, physiological concentrations of free fatty acids significantly stimulate insulin secretion above basal levels in isolated islets, thus supporting a role for free fatty acids as insulin secretagogues.

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