

Dynamic measurements of mitochondrial hydrogen peroxide concentration and glutathione redox state in rat pancreatic β -cells using ratiometric fluorescent proteins: confounding effects of pH with HyPer but not roGFP1

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Keywords

Glucose, HyPer, hydrogen peroxide, mitochondria, mt-AlpHi, oxidative stress, pancreatic beta-cell, pH, roGFP, SypHer

Abbreviations

[pH]_i, intracellular pH; MnTBAP, Mn(III) tetrakis (4-benzoic acid) porphyrin; NAC, N-Acetyl-L-Cysteine; Gn, n mmol/l glucose; roGFP, redox sensitive Green Fluorescent Protein; ROS, reactive oxygen species

Abstract

Using the reactive oxygen species-sensitive fluorescent dyes dichlorodihydrofluorescein and dihydroethidine, previous studies yielded opposite results about the glucose regulation of oxidative stress in insulin-secreting pancreatic β -cells. Here, we used the ratiometric fluorescent proteins HyPer and roGFP1 targeted to mitochondria (mt-HyPer/mt-roGFP1) to monitor glucose-induced changes in mitochondrial hydrogen peroxide concentration (mt-HyPer) and glutathione redox state (mt-roGFP1) in adenovirus-infected rat islet cell clusters. Because of the reported pH-sensitivity of HyPer, the results were compared with those obtained with the mitochondrial pH sensors mt-AlpHi and mt-SypHer. The fluorescence ratio of the mitochondrial probes slowly decreased (mt-HyPer) or increased (mt-roGFP1) in the presence of 10 mmol/l glucose. Besides its expected sensitivity to hydrogen peroxide, mt-HyPer was also highly pH-sensitive. In agreement, changes in mitochondrial metabolism similarly affected mt-HyPer, mt-AlpHi and mt-SypHer fluorescence signals. In contrast, mt-roGFP1 fluorescence ratio was only slightly affected by pH and reversibly increased when glucose was lowered from 10 to 2 mmol/l. This increase was abrogated by the catalytic antioxidant Mn(III) tetrakis (4-benzoic acid) porphyrin but not by N-acetyl-L-cysteine. In conclusion, due to its pH sensitivity, mt-HyPer is not a reliable indicator of mitochondrial hydrogen peroxide in β -cells. In contrast, mt-roGFP1 fluorescence ratio monitors changes in β -cell mitochondrial glutathione redox state with little interference by pH changes. Our results also show that glucose acutely decreases rather than increases mitochondrial thiol oxidation in rat β -cells.

Introduction

Glucose stimulation of insulin secretion by pancreatic β -cells plays a critical role in blood glucose homeostasis. This acute glucose effect depends on the acceleration of its oxidation by glycolysis and the mitochondrial Krebs cycle, with consequent increase in the production of various metabolic coupling factors including NAD(P)H and ATP (reviewed in [1]). This is followed by plasma membrane depolarization, opening of voltage-dependent Ca^{2+} channels, Ca^{2+} influx, and a rise in the free cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) that triggers insulin granule exocytosis (reviewed in [2]). In addition, metabolic coupling factors derived from glucose oxidation amplify the efficacy of cytosolic Ca^{2+} on exocytosis and contribute to the acute stimulation of proinsulin biosynthesis and, in the long term, to the maintenance of the β -cell phenotype (reviewed in [2;3]).

Although the link between the acceleration of glucose metabolism and plasma membrane depolarization involves a rise in the cytosolic ATP/ADP ratio that closes ATP-sensitive K^+ channels, other mechanisms may also be operating [4]. Among these, it has been suggested that an increase in mitochondrial production of hydrogen peroxide (H_2O_2) plays a critical role in glucose stimulus-secretion coupling in β -cells [5]. On the other hand, oxidative stress together with endoplasmic reticulum stress, may contribute to the alteration of β -cell function, survival and gene expression induced by chronic exposure to high glucose and lipid concentrations in the context of type 2 diabetes (reviewed in [6;7]). Such oxidative stress could result from various mechanisms, including increased production of superoxide (coupled to H_2O_2 formation) by the mitochondrial electron transport chain, activation of the NADPH oxidase complex and protein glycation [8-10]. That β -cell oxidative stress is increased by high glucose concentrations was demonstrated using classical detection of oxidative markers such as 4-OH-nonenal and 8-OH-deoxyguanosine on pancreatic sections from type 2 diabetic rodents, and by measuring the oxidation of reactive oxygen species (ROS)-sensitive fluorescent dyes, like dichlorodihydrofluorescein and dihydroethidine, in isolated islets [11;12]. Using the same tools, however, Martens et al. demonstrated that the β -cell ROS concentration is high at low glucose and decreases following nutrient stimulation of mitochondrial metabolism. This is likely because of an increase in NAD(P)H production, hence in ROS scavenging capacity [13], the mechanism of which may include activation of the pentose phosphate pathway [14]. Interestingly, the increase in ROS at low glucose was accompanied by an increase in β -cell apoptosis, and both events were reduced by Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) [13;15]. This antioxidant is usually considered a superoxide dismutase (SOD) and catalase-mimetic and a potent inducer of the antioxidant enzyme heme-oxygenase 1 [16]. However, it has recently been shown that pure MnTBAP mainly reduces peroxynitrite and carbonate radicals while commercial MnTBAP (as used in this study) also contains traces of SOD-mimetic [17;18].

Several methods are frequently used to measure ROS production in living cells, the most popular being the oxidation of Amplex-red and dichlorodihydrofluorescein. Despite their high sensitivity, these probes do not detect specific ROS, undergo rapid photo-oxidation that prevents their use in dynamic measurements, and cannot be targeted to identify the subcellular sites of ROS production (reviewed in [19]). In contrast, new genetically-encoded probes have been developed that allow sensitive and compartment-specific dynamic measurements of ROS production in living cells [20]. Among them, HyPer is a chimaeric protein composed of the H_2O_2 -sensing regulatory domain of the bacterial transcription factor OxyR and a circularly permuted Yellow Fluorescent Protein (cpYFP) insertion [21]. Although HyPer is pH-sensitive as other cpYFP-based probes [21-23], it was recently used to test the subcellular source of oxidative stress in β -cells exposed to saturated fatty acids [24]. In comparison, the redox-sensitive green fluorescent protein (roGFP) is not a specific sensor of H_2O_2 , but is a good indicator of changes in the thiol/disulfide intracellular redox equilibrium [25].

In view of the conflicting data about the glucose regulation of oxidative stress in β -cells, and because of its possible role in β -cell failure in type 2 diabetes, we chose to use genetically-encoded

probes to readdress these questions. In this study, we tested the adequacy of mitochondria-targeted HyPer (mt-HyPer) and roGFP1 (mt-roGFP) to monitor glucose-induced changes in rat β -cell mitochondrial H_2O_2 production and glutathione redox status. To evaluate the influence of mitochondrial pH on these probes, the results were compared with those obtained with the pH sensors mt-AlpHi and mt-SypHer [26;27].

Material and Methods

Chemicals

Na^+ -azide, NH_4Cl and Na^+ -acetate were from Sigma (St-Louis, MI, USA). Mn(III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) was purchased from Alexis Biochemicals (Lausen, Germany) and N-Acetyl-L-Cysteine (NAC) from Merck (Darmstadt, Germany). Restriction enzymes were from Fermentas (St.Leon-Rot, Germany).

Plasmids

The plasmid encoding mt-Hyper [21] was obtained from Evrogen (Moscow, Russia), and that encoding mt-SypHer, an H_2O_2 -insensitive form of HyPer in which cysteine 199 was replaced by a serine [26], was a gift from N. Demaurex (Department of Cell Physiology and Metabolism, University of Geneva Medical School, Geneva, Switzerland). The plasmid encoding doxycycline-inducible mt-AlpHi has been described before [27]. The plasmid encoding mt-roGFP1 (ro1 with mitochondrial targeting sequence in pEGFP-N1, pRA306) [28] was obtained from S.J. Remington (University of Oregon, Eugene, OR, USA).

Adenoviruses

The generation of an adenovirus encoding the fluorescent protein DsRed under the control of the rat insulin promoter (RIP) has been described earlier [29]. Adenoviruses encoding mt-Hyper and mt-roGFP1 were generated and amplified using the AdEasy XL Adenoviral Vector System (Stratagene, La Jolla, CA). Briefly, the cDNA encoding mt-Hyper or mt-roGFP1 were inserted into the pShuttle-CMV vector, and then re-cloned in the adenoviral backbone plasmid pAdEasy. The resultant pAd-mt-Hyper and pAd-mt-roGFP1 plasmids were digested with PacI and transfected into HEK293 cells to generate adenovirus particles. Adenoviruses encoding RIP-mt-AlpHi and mt-SypHer were generated and amplified using the Adeno-X Adenoviral Expression System (Clontech, Mountain View, CA). Briefly, the promoter sequence for doxycycline-inducible expression (tetON CMVmin) was removed from the pTRE Shuttle vector (Clontech) and replaced by the RIP followed by mt-AlpHi, or by a CMV promoter together with mt-SypHer. The resulting constructs were re-cloned into the pAdenoX vector. The vector was linearized using PacI to be used for transfection of HEK293 cells to generate adenovirus particles. All adenoviruses were amplified in HEK293 cells and purified on CsCl gradient.

Islet isolation and adenoviral infection

All animal experimentations were approved by the local Institutional Committee on Animal Experimentation of the Faculty of Medicine of the Université catholique de Louvain (Project UCL/MD/2009/009). Islets were isolated by collagenase digestion of the pancreas of male Wistar rats (180-200 g), as previously described [30]. They were washed and hand-picked under a stereomicroscope to ensure high purity of the preparation, then dispersed into small cell clusters using trypsin and gentle pipetting in a Ca^{2+} -free medium. These clusters were plated on glass coverslips and cultured at 37°C in the presence of 5% CO_2 with RPMI 1640 medium (Invitrogen,

Carlsbad, CA) containing 10 mmol/l glucose (G10), 10% FBS, penicillin and streptomycin. After overnight culture, cells were infected with 1 to 1.5 μ l of RIP-DsRed, mt-HyPer, mt-roGFP1, mt-SypHer, or RIP-mtAlphi encoding adenovirus. They were then cultured for 2 days before fluorescence measurements.

Static fluorescence measurements

Two days after co-infection of islet cell clusters with RIP-DsRed plus mt-HyPer, mt-roGFP1, mt-SypHer, or RIP-mt-AlpHi, cell nuclei were stained for 20 min with the DNA-binding fluorescent dye Hoechst 33342. The fluorescence was analyzed using EVOS microscope (Bothell, WA) and the following excitation/emission wavelengths (nm): Hoechst 33342 (357/447); DsRed (531/593); mt-HyPer, mt-roGFP1 and mt-SypHer (470/525). The images were captured using a 40x objective. In other experiments, the mitochondrial expression of the various probes was documented with a Nikon Eclipse TE2000-E inverted microscope equipped with a confocal QLC100 spinning disk (Visitech International, Sunderland, UK), using a 60x oil immersion objective.

Dynamic fluorescence measurements

After culture, the coverslip was mounted at the bottom of a 37°C temperature-controlled chamber place on the stage of an inverted microscope. Cell clusters were perfused at a flow rate of ~1 ml/min with a bicarbonate-buffered Krebs solution containing (mmol/l) NaCl (120), KCl (4.8), CaCl₂ (2.5), MgCl₂ (1.2), NaHCO₃ (24), 1 g/l BSA (fraction V, Roche, Basel, Switzerland), and various glucose concentrations and test substances. This solution was continuously gassed with O₂/CO₂ (94/6) to maintain pH ~7.4. Excitation light at appropriate wavelengths was produced by a monochromator: 495/420 nm for mt-HyPer, 400/480 nm for mt-roGFP1, 480/430 nm for mt-SypHer, and 490 nm for RipMtAlpHi. For all probes, a 535 nm emission filter was used. The cells were analyzed with a 40x objective and images were acquired every 30 s. Results are shown as means $\pm$ SE for the indicated number of cells and experiments. In each figure, the statistical significance of changes in fluorescence ratio (or absolute levels for mt-AlpHi) between selected time points (one minute average) was assessed by one-way ANOVA for repeated measurements followed by a test of Newman-Keuls.

Results

The CMV-promoter drives the expression of the mitochondrial sensors preferentially in β -cells

Dissociated rat islet cells were infected with adenoviruses carrying transgenes expressed under the control of the CMV promoter. The proportion of β -cells and non β -cells expressing CMV-driven mt-roGFP1, mt-HyPer and mt-SypHer was determined by co-infection with an adenovirus encoding DsRed under the control of the rat insulin promoter (RIP-DsRed) (Fig. 1) [29]. On average, 84% of mt-HyPer-positive cells were also positive for RIP-DsRed, while double positivity was observed in 90% of mt-roGFP1-positive cells and 80% of mt-SypHer-positive cells. These results demonstrate that mt-HyPer, mt-roGFP1 and mt-SypHer are preferentially expressed in β -cells vs. non β -cells. In addition, the fluorescence intensity of the probes was lower in RIP-DsRed-negative vs. RIP-DsRed-positive cells (compare the cluster and the single non β -cell indicated by an arrow in Fig. 1). Therefore, to further reduce the likelihood of studying non β -cells, changes in probe fluorescence ratios were only measured in brightly fluorescent cells within clusters. As expected, all RIP-mt-AlpHi-positive cells were also positive for RIP-DsRed (data not shown). Confocal imaging of mt-HyPer, mt-roGFP1 and mt-SypHer expressing cells confirmed that these probes correctly targeted to mitochondria (Fig. 1, right panels).

Glucose-induced changes in mt-Hyper signal

To evaluate whether glucose acutely increases mitochondrial H_2O_2 production in rat β -cells, we initially tested the effects of glucose on mt-Hyper fluorescence ratio in perfused islet cell clusters. When the medium contained 10 mmol/l glucose (G10) throughout the experiment, mt-Hyper fluorescence ratio tended to decrease slowly for at least 1 h (Fig. 2A). As expected, addition of 100 $\mu\text{mol/l}$ H_2O_2 to G10 rapidly increased this ratio 2 to 3-fold. When the glucose concentration was increased stepwise from G2 to G30, mt-Hyper fluorescence ratio significantly increased by about 20% between G2 and G10, but did not further increase between G10 and G30 (Fig. 2B). On the contrary, when glucose was reduced from G10 to G2, mt-Hyper fluorescence ratio transiently increased then decreased to a lower level (Fig. 2C). Upon return to G10, mt-Hyper fluorescence ratio increased back to its initial level. At first sight, these glucose-induced changes in mt-Hyper fluorescence ratio could be taken as evidence that the glucose stimulation of pancreatic β -cells increases mitochondrial H_2O_2 concentration from G2 to G10, with no further increase above G10. However, the catalytic antioxidant MnTBAP and the free radical scavenger NAC failed to suppress glucose-induced changes of the mt-Hyper fluorescence (Fig. 2D-E). These findings suggested that during glucose stimulation of rat islet β -cells, mt-Hyper fluorescence is altered by a mitochondrial parameter other than the H_2O_2 concentration.

Glucose-induced changes in mt-Hyper fluorescence ratio mainly reflect changes in mitochondrial pH

It has been reported that, like other fluorescent probes derived from circularly permuted fluorescent proteins, Hyper is sensitive to changes in intracellular pH [21]. The similarity between the glucose-induced changes in mt-Hyper signal (see Fig. 2) and mitochondrial pH increase measured with mt-AlpHi in rat islets [27] suggested that these changes in mt-Hyper fluorescence ratio in β -cells could reflect changes in mitochondrial pH rather than H_2O_2 .

To test this hypothesis, we first measured the effects of cell acidification induced by Na^+ -acetate and of cell alkalinization with NH_4Cl in islet cells expressing mt-Hyper and compared the results to those obtained in cells expressing the mitochondrial pH-sensor mt-AlpHi or mt-SypHer. The latter pH probe is derived from Hyper by replacing the first H_2O_2 -sensing cysteine 199 by a serine, as previously described [26]. As shown in figure 3 (panels A, C and E), Na^+ -acetate similarly decreased while NH_4Cl similarly increased mt-Hyper fluorescence ratio, mt-AlpHi fluorescence intensity and mt-SypHer fluorescence ratio. As expected, subsequent addition of 100 $\mu\text{mol/l}$ H_2O_2 to G10 only increased mt-Hyper fluorescence ratio without affecting mt-SypHer or mt-AlpHi signals (Fig. 3A, C and E).

Inhibitors of the electron transport chain are considered to increase mitochondrial ROS production. It has, however, also been reported that they induce mitochondrial matrix acidification in pancreatic β -cells and in other cell types [26;27]. In agreement with the latter effect, addition of 3 mmol/l azide to G10 similarly decreased mt-Hyper fluorescence ratio, mt-AlpHi fluorescence intensity and mt-SypHer fluorescence ratio (Fig. 3B, D and F). This effect of azide was completely reversible.

We finally tested the effects of glucose on β -cell mitochondrial pH in our experimental system. In agreement with earlier data showing that glucose stimulation triggers mitochondrial matrix alkalinization in rat islets [27], both mt-SypHer and mt-AlpHi signals increased when the glucose concentration was raised from G2 to G10. A further increase was observed between G10 and G30 with mt-AlpHi but not mt-SypHer (Fig. 4). The similarity of the latter changes with those observed in mt-Hyper-expressing cells strongly supported our hypothesis that glucose-induced changes in mt-Hyper fluorescence ratio in β -cells better reflect changes in mitochondrial pH than in H_2O_2 concentration.

Acute glucose-induced changes in mt-roGFP1 fluorescence

As mt-HyPer proved highly sensitive to changes in intracellular pH, we decided to test whether roGFP1 targeted to mitochondria (mt-roGFP1) could be used to monitor glucose-induced changes in β -cell mitochondrial redox state independently from changes in pH. In contrast with HyPer, the redox-sensitive probe mt-roGFP1 does not directly sense H_2O_2 but rather reflects changes in the thiol/disulfide (GSH/GSSG) ratio [20;28]. The pH-sensitivity of mt-roGFP1 was first assessed using Na^+ -acetate and NH_4Cl . As shown in figure 5A, mt-roGFP1 fluorescence ratio was not affected by Na^+ -acetate and only slightly increased upon cell alkalization with NH_4Cl , demonstrating a very low pH sensitivity of this probe. Consistent with these findings, mt-roGFP1 fluorescence ratio was not significantly affected by azide (Fig. 5B). In contrast, mt-roGFP1 signals rapidly increased upon addition of $100\ \mu\text{mol/l}$ H_2O_2 , confirming that the probe can detect an increase in H_2O_2 concentration in the mitochondrial matrix. We next tested the effects of glucose in islet cell clusters expressing mt-roGFP1. As shown in figure 6 (panels A and B), mt-roGFP1 fluorescence ratio tended to increase slowly and regularly during perfusion with G10 and was unaffected by a rise in glucose from G10 to G30. However, in contrast with results obtained with the pH sensors mt-AlpHi and mt-SyPher, mt-roGFP1 fluorescence ratio reversibly increased when glucose was acutely lowered from G10 to G2 and raised back to G10 (Fig. 6C). Remarkably, the effect of G2 was almost fully suppressed by addition of MnTBAP, but not NAC, to the perfusion medium (Fig. 6D and E), indicating that glucose stimulation acutely suppresses rather than increases thiol oxidation in β -cell mitochondria.

Discussion

In this study, we demonstrate that, due to its high pH sensitivity, mt-HyPer is not a reliable tool to measure nutrient-induced changes in mitochondrial H_2O_2 production in insulin-secreting pancreatic β -cells. In contrast, mt-roGFP1, which displays low pH sensitivity, can be used to monitor nutrient-induced changes in β -cell mitochondrial redox status. The results obtained with mt-roGFP1 suggest that lowering the glucose concentration from 10 to 2 mmol/l acutely increases thiol (most likely GSH) oxidation in the mitochondrial matrix of β -cells, whereas increasing glucose from 10 to 30 mmol/l does not detectably affect it within 40 min.

In comparison with small chemical fluorescent probes such as dichlorodihydrofluorescein and dihydroethidine that distribute throughout the cell and are not measuring a specific type of ROS species [19], genetically-encoded redox-sensitive probes derived from fluorescent proteins are attractive probes as they allow monitoring of compartment-specific redox changes (reviewed in [20]). In their initial report on HyPer, Belousov et al. demonstrated that the probe was sensitive to small changes in intracellular H_2O_2 while being insensitive to other oxidants such as superoxide, peroxynitrite, and oxidized glutathione [21]. However, as expected for probes derived from circularly-permuted fluorescent proteins [31], HyPer fluorescence ratio was also reported to be sensitive to pH variations as low as 0.2 pH units, the fluorescence ratio of its reduced form displaying a ~ 1.8 -fold increase upon alkalization from pH 7.1 to 7.4 and a $\sim 30\%$ decrease upon acidification from pH 7.1 to 6.9 [21]. In agreement, concentrations of Na^+ -acetate and NH_4Cl previously reported to affect mitochondrial pH by ~ 0.2 units in HeLa cells [32], respectively decreased by $\sim 20\%$ and increased ~ 1.8 -fold the mt-HyPer fluorescence ratio in rat islet cells. Nevertheless, HyPer has been used to monitor H_2O_2 production in many cell types under physiological and pathophysiological conditions, including in clonal insulin-producing cells and primary β -cells cultured under lipotoxic conditions, without adequately assessing the possible confounding effects of changes in pH [20;21;24].

It has previously been shown that the stimulation of pancreatic islets with glucose and other nutrients induce rapid changes in their cytosolic and mitochondrial pH, the latter being of larger amplitude than the former [27;33;34]. Using 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein, Shepherd and Henquin demonstrated that the intracellular pH of mouse β -cells is slightly acidic (pH

6.9) in the absence of glucose, and that their stimulation with glucose and other mitochondrial substrates induces a net alkalization of ~0.2 pH units that results from a balance between the alkalizing action of an increase in mitochondrial metabolism and the acidifying action of a rise in cytosolic calcium concentration [33;34]. Using the mt-AlpHi pH probe, it was shown that the mitochondrial matrix of rat pancreatic β -cells is unusually acidic at low glucose concentration (pH~7.25) and that glucose stimulation alkalizes the mitochondrial matrix to a value close to that measured in other cell types (pH ~7.7) [27]. This alkalization, which was correlated with the increase in cytosolic ATP concentration, was proposed to control mitochondrial ATP generation during sustained stimulation of insulin secretion [35].

We observed that, although mt-HyPer is able to detect an increase in β -cell mitochondrial H_2O_2 , the changes in mt-HyPer fluorescence ratio induced by glucose, Na^+ -acetate, NH_4Cl and azide were almost identical to those observed using the pH sensors mt-AlpHi and mt-SypHer. In addition, the glucose-induced changes in mt-HyPer fluorescence ratio were not affected by the antioxidants MnTBAP and NAC, suggesting that they do not result from changes in H_2O_2 concentration. Altogether, our results indicate that the unusual change in mitochondrial matrix pH observed in β -cells stimulated with nutrients combined with the high pH sensitivity of HyPer precludes the use of that probe to measure changes in H_2O_2 concentration under these conditions. Whether this probe can be used in the cytosol of β -cells was not tested in the present study, but the amplitude of glucose-induced changes in cytosolic pH is large enough to affect HyPer fluorescence ratio [33]. Therefore, we suggest that, when using HyPer in living cells, SypHer be systematically used to control for the possible confounding effect of changes in pH.

In comparison with HyPer, the fluorescent protein roGFP1 is not selective for H_2O_2 but specifically senses changes in glutathione redox state, which results from the dynamic equilibrium between its oxidation and reduction. This probe therefore only indirectly senses an increase in H_2O_2 levels. However, roGFP1 has the advantage of being much less sensitive than HyPer to changes in pH between 6.0 and 8.0 [20], as confirmed by our data. To our knowledge, the use of roGFP1 in native β -cells or β -cell lines has not been reported yet. We observed that lowering the glucose concentration from 10 to 2 mmol/l rapidly and reversibly increased mt-roGFP1 fluorescence ratio and that this effect was prevented by the antioxidant MnTBAP but not NAC. In contrast, an increase in glucose from 10 to 30 mmol/l did not detectably affect mt-roGFP1 signal within 40 min of stimulation. These results question previous studies showing that glucose stimulation acutely increases β -cell ROS production [12;36]. On the contrary, they extend previous studies demonstrating that the oxidation of dichlorodihydrofluorescein and dihydroethidine in β -cells was high in the absence of glucose and rapidly decreased between 0 and 10 mmol/l glucose, but did not increase upon a rise in glucose from 10 to 20 mmol/l [13-15]. In these studies also, the non-specific redox catalyst MnTBAP [17;18;37] significantly reduced β -cell ROS production and apoptosis induced by low glucose. In contrast, the free radical scavenger and GSH precursor NAC did not prevent the increase in mt-roGFP1 signal induced at low glucose, nor did it protect β -cells against apoptosis under these conditions (our unpublished results). The different efficacy of MnTBAP and NAC on both ROS production and apoptosis at low glucose might be related to the type of ROS generated, their site of production, or the ability of these compounds to reach the mitochondrial matrix. However, overexpression of cytosolic and mitochondrial antioxidant enzymes with defined ROS-specificities will be required to correctly address these questions.

Previous studies have yielded conflicting results regarding the glucose regulation of β -cell ROS production (reviewed in [38]). The present study reinforces the view that acute lowering of the glucose concentration from 10 to 2 mmol/l reversibly increases β -cell ROS production and/or reduces their antioxidant defense [14;15;38]. The fact that high glucose (30 mmol/l) did not acutely increase mt-roGFP1 oxidation in islet cell clusters does not, however, exclude the possibility that high glucose acutely stimulates ROS production in a compartment from where it does not reach the mitochondrial matrix, or that it does so only transiently, thereby escaping detection by roGFP1.

They also do not rule out the hypothesis that a long-term exposure to high glucose concentrations (30 vs. 10 mmol/l) could induce mitochondrial oxidative stress.

In conclusion, the high pH sensitivity of mt-HyPer prevents its use as a reliable indicator of glucose-induced changes in mitochondrial H₂O₂ production in rat β -cells. In contrast, mt-roGFP1 can be used to monitor changes in glutathione redox state in β -cell mitochondria independently from changes in pH. Our results also show that glucose acutely reduces rather than increases mitochondrial thiol oxidation in rat β -cells.

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Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

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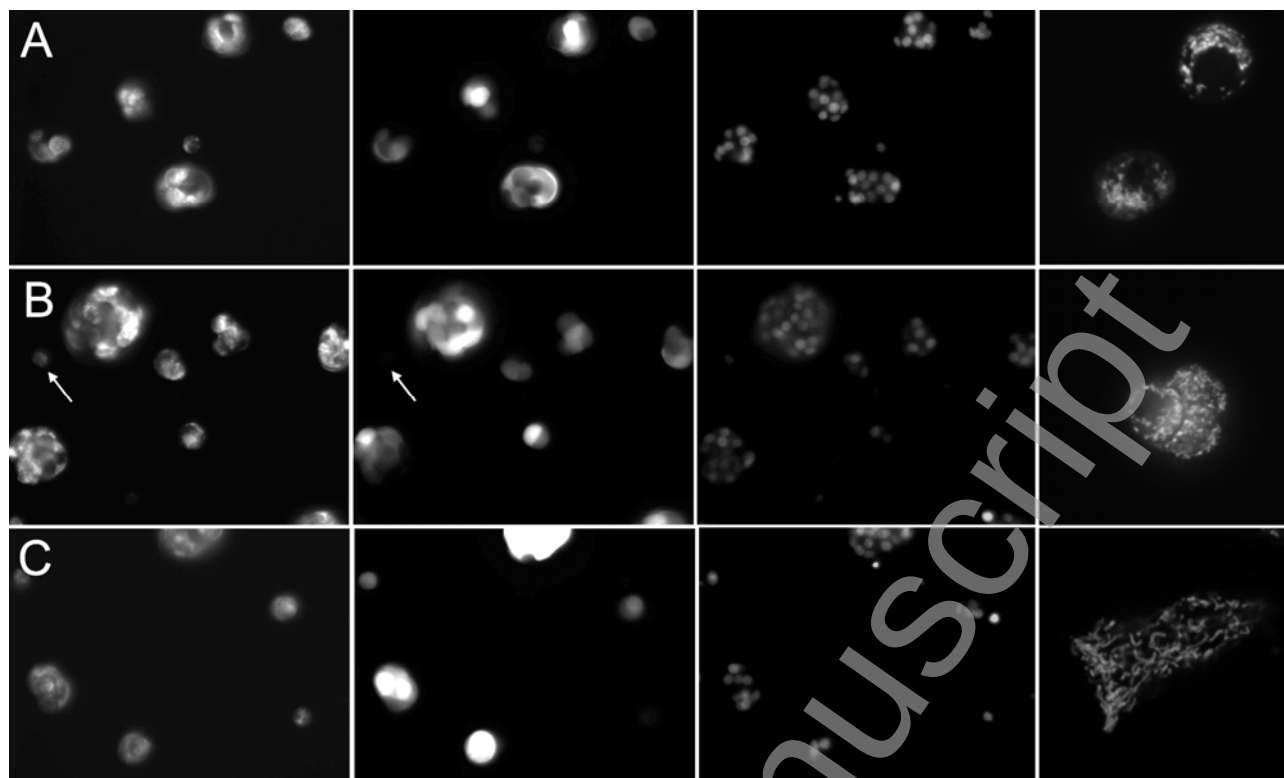


Figure 1. Expression of mt-HyPer, mt-roGFP1, and mt-SypHer with RIP-DsRed in rat islet cell clusters and their intracellular localization. Two days after double adenoviral infection, the fluorescence of islet cell clusters was captured as described in Methods. Left panels, mt-HyPer (A), mt-roGFP1 (B) or mito-SypHer (C) fluorescence. Middle left panels, β -cells identified by their RIP-DsRed fluorescence. Middle right panels, Hoechst 33342 fluorescence. Right panels, confocal fluorescent images of islet cell clusters expressing the redox-probes or of an INS1 cell expressing mt-SypHer. The intracellular distribution of the probes is characteristic of mitochondrial localization. The arrow in B points to RIP-DsRed-negative cells with weak expression of the other fluorescent probe. Fluorescence intensity data were converted to pseudocolor images. Data are representative for 3 experiments.

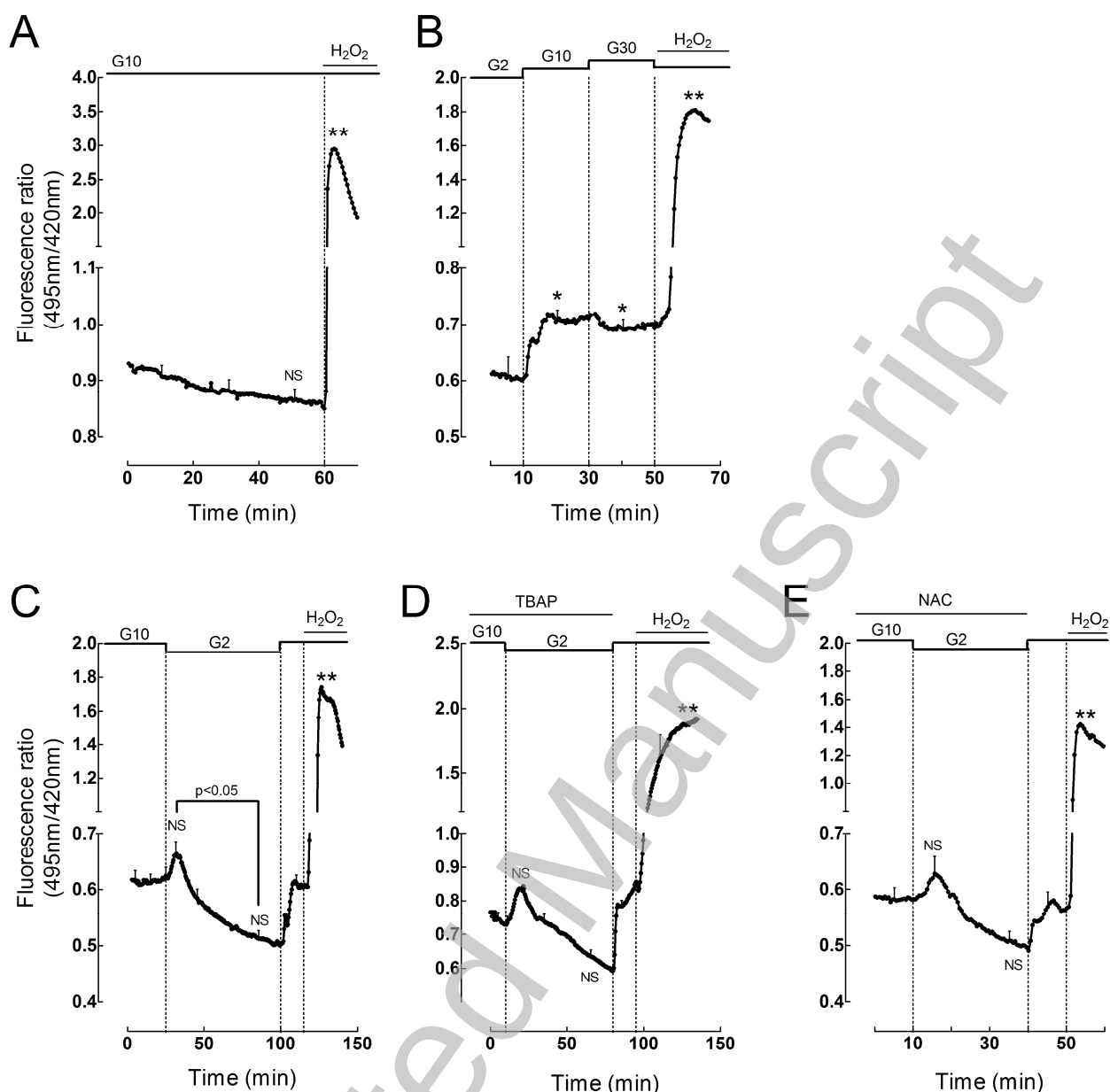


Figure 2. Glucose-induced changes in mt-HyPer fluorescence ratio in rat islet cell clusters.

Two days after adenoviral infection, mt-HyPer fluorescence ratio was measured in rat islet cell clusters perfused with a medium containing 10 mmol/l glucose (G10) throughout the experiment (A) or various glucose concentrations under control conditions (B-C) or in the presence of 100 μ mol/l MnTBAP (D) or 1 mmol/l NAC (E), as shown on top of the panels. At the end of each experiment, the antioxidant was withdrawn and maximal oxidation of HyPer was triggered by addition of 100 μ mol/l H_2O_2 . Data are means $\pm$ SE for at least 3 experiments. NS, * and **, not significant, $P<0.05$ and $P<0.001$ vs. initial period (min 1 to min 6) of perfusion at the time indicated.

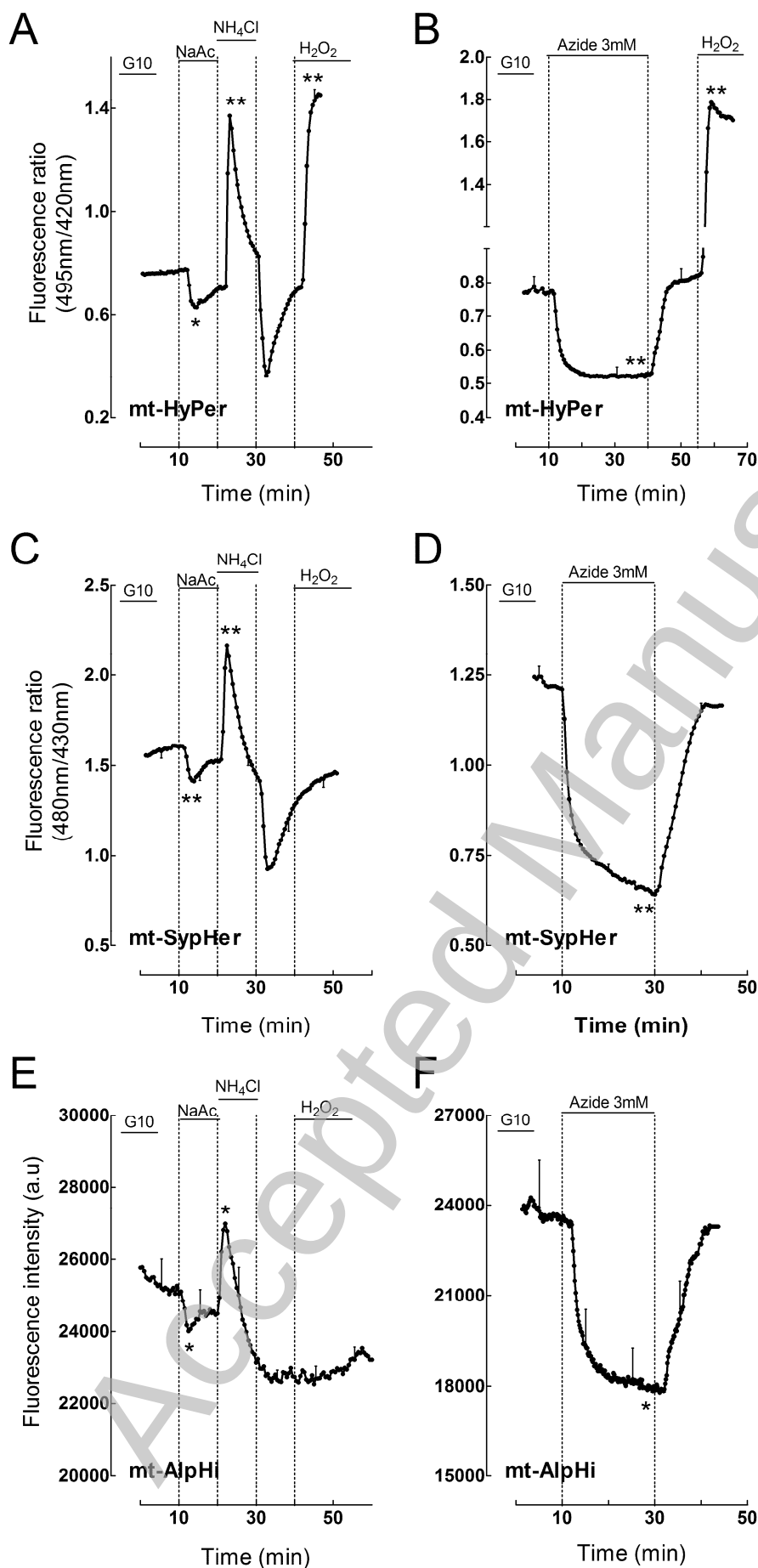


Figure 3. pH-sensitivity of mt-Hyper, mt-SypHer and mt-AlpHi fluorescence signal in rat islet cell clusters. Two days after adenoviral infection, rat islet cell clusters expressing mt-Hyper (A, B), mt-SypHer (C, D) or mt-AlpHi (E, F) were perfused with a medium containing 10 mmol/l glucose throughout the experiment with sequential addition of 30 mmol/l Na⁺-acetate (NaAc) and 30 mmol/l ammonium chloride (NH₄Cl) (A, C, E), or with addition of 3 mmol/l sodium azide (B, D, F) as indicated on top of the panels. At the end of each experiment depicted in panels A-C and E, the H₂O₂ sensitivity of the probe was tested by addition of 100 μ mol/l H₂O₂ to the medium. Data are means $\pm$ SE for at least 3 experiments. * and **, $P < 0.05$ and $P < 0.001$ vs. initial period (min 1 to min 6) of perfusion at the time indicated.

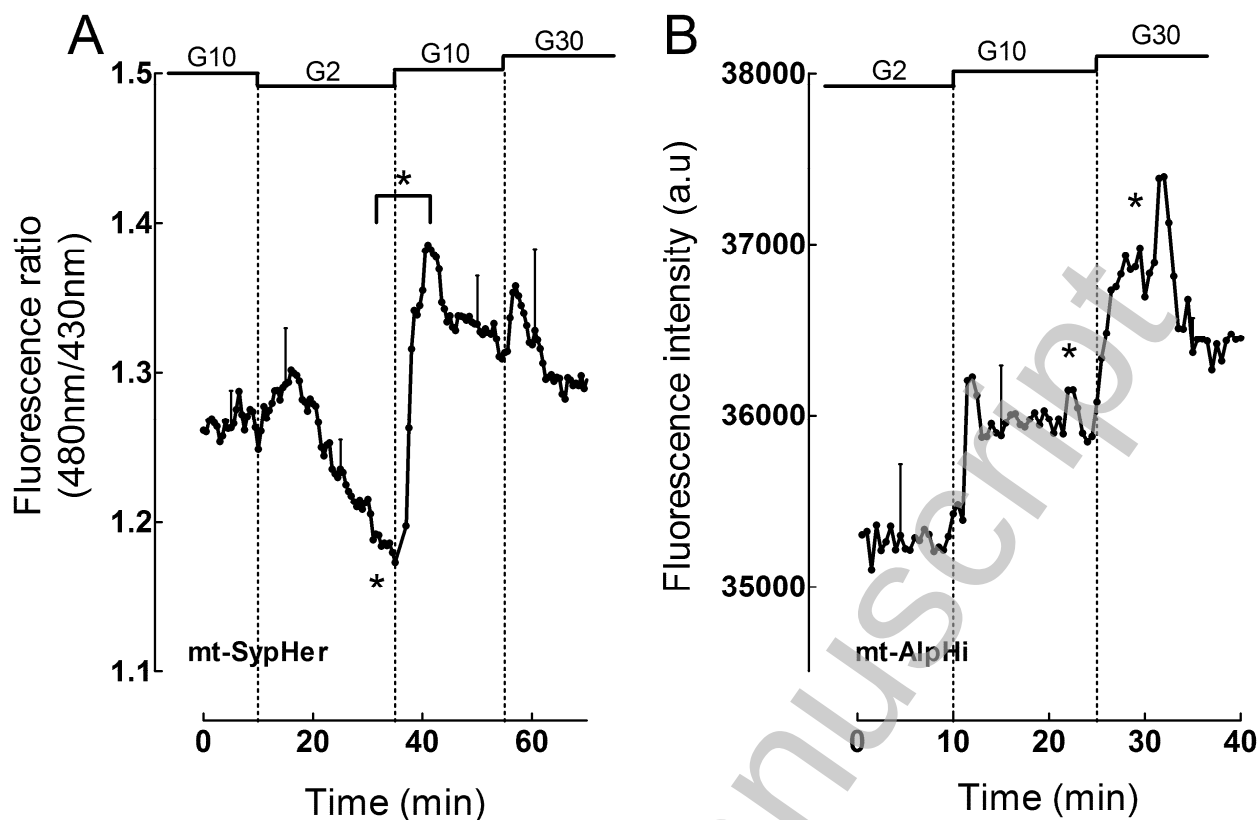


Figure 4. Effects of glucose on mt-SypHer and mt-AlpHi fluorescence signal in rat islet cell clusters. Two days after adenoviral infection, rat islet cell clusters expressing mt-SypHer (A) or mt-AlpHi (B) were perfused with a medium containing various glucose concentrations as indicated on top of the panels. Data are means $\pm$ SE for at least 3 experiments. *, $P < 0.05$ vs. initial period (min 1 to min 6) of perfusion at the time indicated.

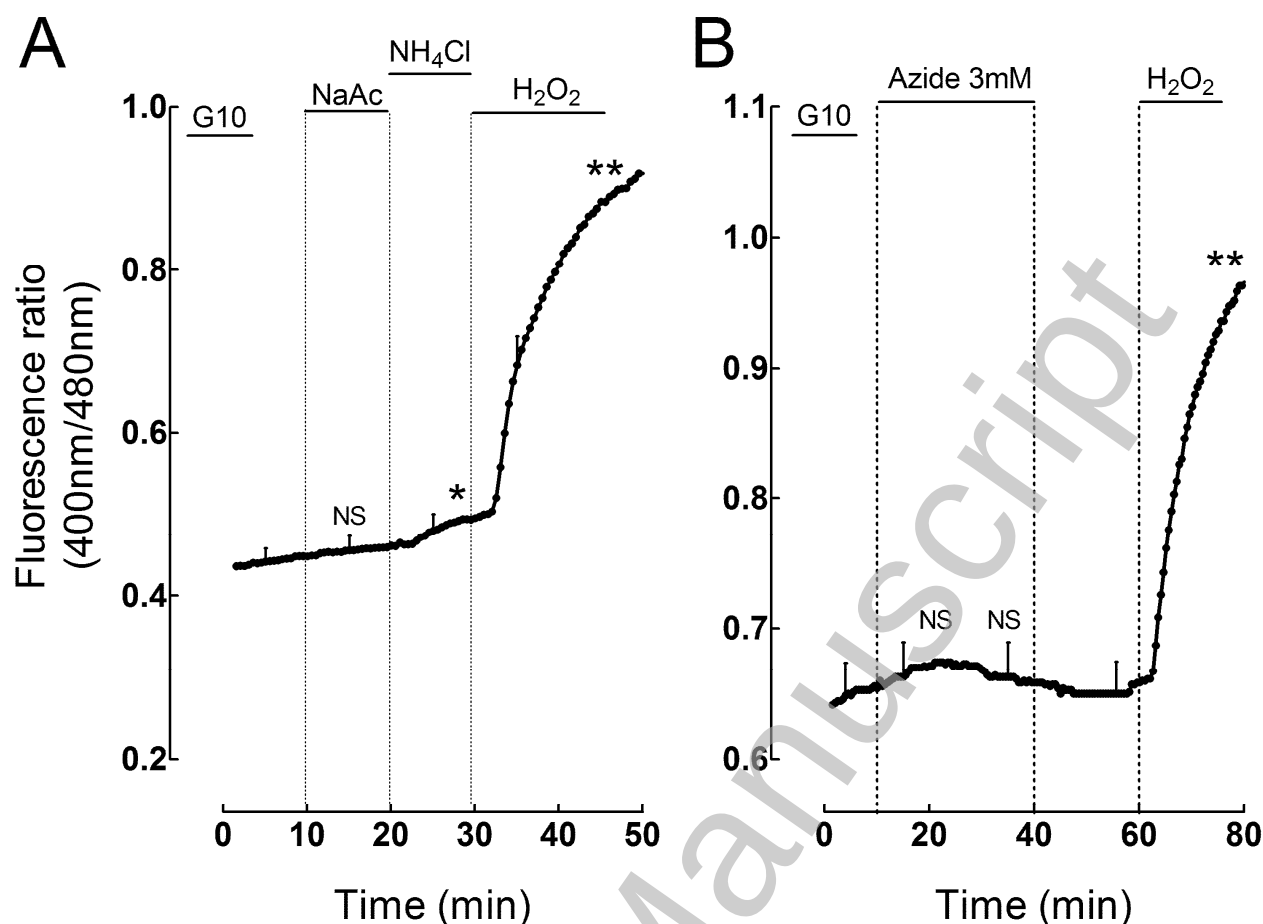


Figure 5. Effects of cell acidification and alkalization in rat islet cell clusters expressing mt-roGFP1. Two days after adenoviral infection, rat islet cell clusters expressing mt-roGFP1 were perfused with a medium containing 10 mmol/l glucose throughout the experiment with sequential addition of 30 mmol/l NaAc and 30 mmol/l NH_4Cl (A), or of 3 mmol/l azide (B), as indicated on top of the panels. At the end of each experiment, 100 $\mu\text{mol/l}$ H_2O_2 was added resulting in maximal oxidation of mt-roGFP1. Data are means $\pm$ SE for at least 3 experiments. NS, * and **, not significant, $P < 0.05$ and $P < 0.001$ vs. initial period (min 1 to min 6) of perfusion at the time indicated.

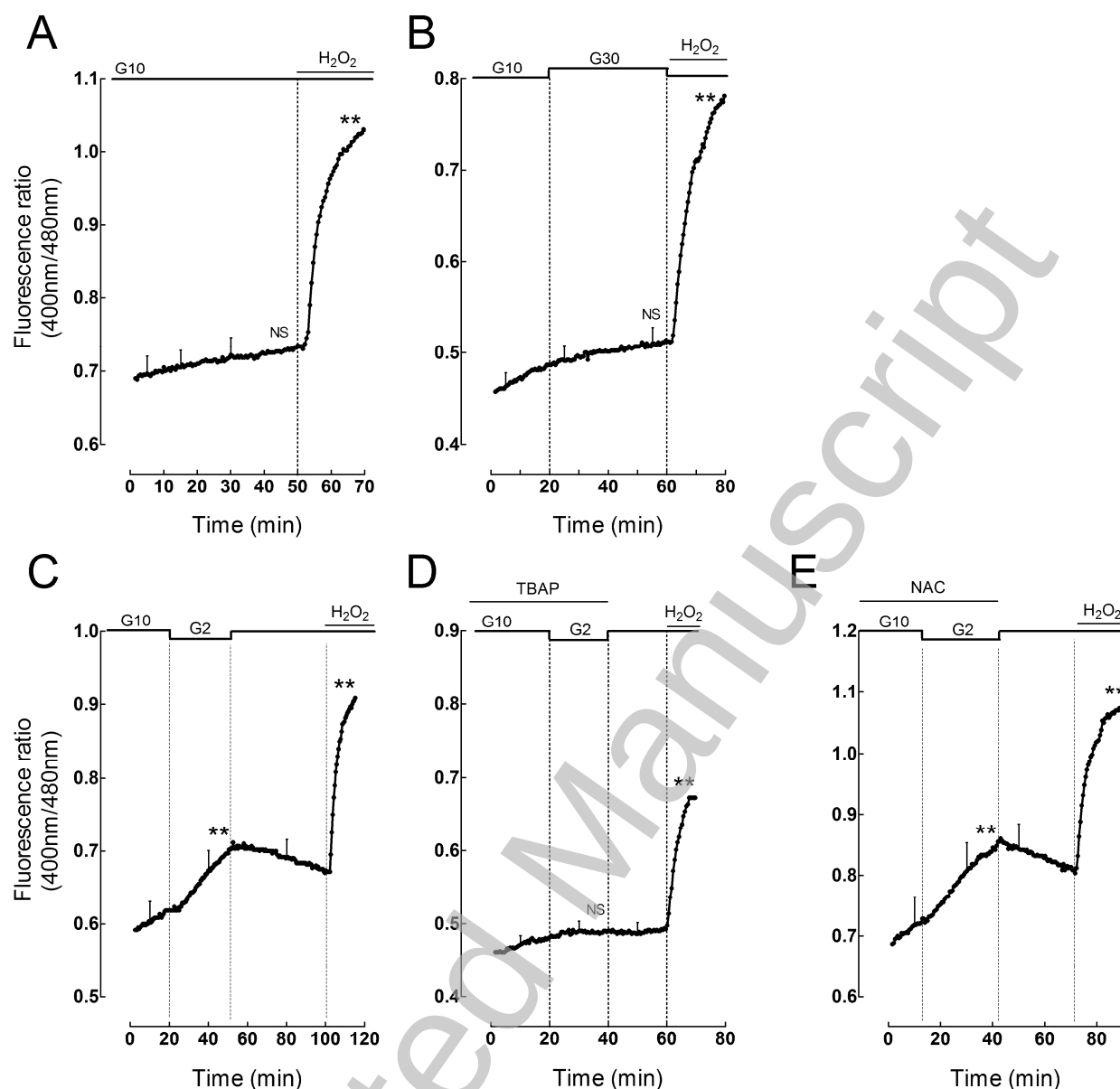


Figure 6: Effects of glucose in rat islet cell clusters expressing mt-roGFP1. A, mt-roGFP1 fluorescence ratio was measured in islet cell clusters perfused with a medium containing G10 (A). B-C, the glucose concentration was changed from G10 to G30 (B) or from G10 to G2 (C). D-E, cells were pretreated with the antioxidant MnTBAP (100 μ mol/l; D) or NAC (1 mmol/l; E) before the reduction in glucose concentration from G10 to G2. At the end of each experiment, 100 μ mol/l H_2O_2 was added resulting in maximal oxidation of mt-roGFP1. Data are means $\pm$ SE for at least 3 experiments. NS and **, not significant and $P < 0.001$ vs. initial period (min 1 to min 6) of perfusion at the time indicated.