CALCINEURIN ACTIVITY AS AN INDICATOR OF OXIDATIVE STRESS IN NORMAL ISLET CELLS AND INSULINOMA CELLS

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ABSTRACT

A comparative study was conducted to evaluate calcineurin activity in normal pancreatic beta cells and insulinoma cells in relation to their oxidative state. In comparison to normal islets, insulinoma cells had enhanced oxidative stress as evidenced by increased content of thiobarbituric acid reactive substances. In addition, diminished activity of calcineurin in insulinoma cells was concomitant with decreased content of reduced glutathione and glutathione peroxidase activity signifying diminished antioxidant status in these cells. Culturing insulinoma cells in presence of the calcineurin inhibitor cyclosporin A resulted in further decrease of calcineurin activity with restoration of glutathione peroxidase but without restoration of reduced glutathione levels. These results indicate that an estimate of oxidative stress in pancreatic islets and insulinoma cells can be obtained by assaying calcineurin activity.

KEY WORDS

Calcineurin; Cyclosporin; Glutathione peroxidase; Insulin; Insulinoma; Oxidative stress.

INTRODUCTION

Calcineurin (CaN; MW 80 kDa; Ca²⁺-calmodulin dependent protein phosphatase 2B) mediates the second messenger functions of intracellular Ca²⁺ by reversible dephosphorylation of target proteins (1,2). Further, Fe³⁺ in the binuclear center of calcineurin is susceptible to free radicals resulting in inactivation of the enzyme (3) denoting that its physiological functions may be targeted under disease conditions wherein oxidative stress is encountered. One such mechanism concerns the regulation of pancreatic β cell functioning wherein the synthesis and release of insulin are reportedly regulated by calcineurin. Not only is the presence of calcineurin in pancreatic cells established (4), but it has been reported recently that secretagogues that elevate cytosolic Ca²⁺ levels in pancreatic β cells can also activate calcineurin to regulate insulin gene transcription involving pathways similar to those involved in the activation of T-lymphocytes (5). The present communication describes a comparative account of calcineurin and oxidative stress related

parameters between normal pancreatic and insulinoma cells and suggests that calcineurin may regulate the functioning of pancreatic cells and insulin metabolism in these cells primarily by 'sensing' the oxidative stress encountered in them.

MATERIALS AND METHODS

Materials

Thiobarbituric acid, trifluoperazine, reduced form of nicotinamide adenine dinucleotide (NADH), phenazine methosulphate, sodium salt of nitro blue tetrazolium, p-nitrophenyl phosphate (p-NPP), cumene hydroperoxide, o-phthalaldehyde (OPT), collagenase, Ficoll, and cyclosporin A (CsA), were obtained from Sigma Chemical Co, USA. RPMI 1640 medium was obtained from Gibco- BRL, USA. All other chemicals of analytical grade were obtained from local suppliers.

Isolation of pancreatic islets and culture of insulinoma cells

Pancreatic islet cells were isolated from healthy male Wistar rats ($\approx$ weight 200 g) by collagenase digestion followed by centrifugation on Ficoll gradient as described by Shibata *et al.*, (6). Rat Insulinoma cell line (RIN) was obtained from National Centre for Cell Science, Pune, India and cultured in RPMI

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1640 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml) in an incubator in presence of 5 % CO₂ at 37°C as described by Afsari *et al* (7). Insulinoma cells (3x10⁶ cells) were harvested by trypsinization and precipitated by centrifugation at 900 x g for 5 minutes. The cells were homogenized in ice-cold homogenization buffer (50mM Tris-HCl pH 7.4, 2mM EDTA, 10mM EGTA, 1mM phenylmethylsulfonylfluoride, 1mM dithiothreitol, 100-µM leupeptine, and 0.3-µM aprotinin) using a Teflon glass homogenizer. Homogenates were clarified by centrifugation at 10,000x g for 10 minutes and supernatants were analyzed for various parameters described below after determining protein contents in them as per Bradford (8).

Determination of metabolites

Thiobarbituric acid reactive substances (TBARS) were estimated as described by Sadasivudu *et al* (9) employing malondialdehyde as the reference standard. Contents of reduced and oxidized forms of glutathione (GSH and GSSG respectively) were measured as per Hissin and Hilf (10).

Enzyme assays

Calcineurin activity was determined as described by Padma and Subramanyam, involving the assay of trifluoperazine-sensitive, calmodulin-dependent protein phosphatase activity (11). One unit of calcineurin activity is defined in terms of µ moles of p-nitrophenol liberated per mg protein per minute. Superoxide dismutase (SOD) was assayed according to Kakkar *et al* (12). One unit of the enzyme activity denotes the enzyme required to effect 50% inhibition of the reduction of nitrobluetetrazolium in one minute under the assay conditions. Glutathione peroxidase (GPx) was assayed as per Martinez *et al* (13). One unit of GPx represents µg of GSSG formed per mg protein.

RESULTS AND DISCUSSION

The present study was conducted to compare oxidative stress in normal pancreatic islets with that experienced in insulinoma cells as well as to correlate oxidative stress with calcineurin, which mediates insulin metabolism. Such an investigation was in continuation of earlier studies from this laboratory, which reported decreased activity of serum calcineurin under conditions of oxidative stress as encountered in chronic renal failure (14)

and acute leukemia (15).

Data obtained by comparing normal pancreatic islets with RIN cells indicated the oxidative stress encountered in insulinoma cells with significant accumulation of thiobarbituric acid reactive substances (TBARS) and decreased contents of reduced glutathione (GSH) in these cells with decreased GSH/GSSG ratio (Table 1). The contents of TBARS were increased (68% increase) and those of GSH decreased (76% decrease) in insulinoma cells as compared to the normal cells, even though SOD activity in them was comparable to that found in normal pancreatic islets. It is known that insulinoma cells intrinsically have a low capacity to maintain intracellular GSH in absence of extracellular thiol supplementation (16). Also, insulinoma cells have weak anti-oxidative defense systems and possess lesser activity of glutathione peroxidase that is required for H₂O₂ degradation (17). Since the activities of SOD were relatively the same in RIN cells as well as in normal pancreatic islets, it may be possible that glutathione peroxidase may be the main enzyme involved in protecting the cells from oxidative stress. In this regard, it is worth recollecting that beta cells are undersupplied with well-characterized antioxidant enzymes but possess an additional antioxidant defense system, in the form of peroxiredoxins that are inducible by oxidative as well as nitrosative stress factors (18).

Data shown in Table 1 also demonstrates that calcineurin activity is decreased in RIN cells in comparison to the values obtained in normal pancreatic islets. This decreased activity could be attributed to the susceptibility of Fe³⁺ in the dinuclear metal binding center (in the active site) of calcineurin to inhibition by reactive oxygen species that accumulate under conditions of oxidative stress (3) and is reminiscent of earlier reports from this laboratory in chronic renal failure (14) and acute leukemia (15) wherein oxidative stress conditions are known to prevail.

Culturing insulinoma cells in presence of CsA, a specific inhibitor of calcineurin indicated the significance of calcineurin in relation to oxidative stress in these cells. Even though the diabetogenic action of CsA has been ascribed to decrease in insulin mRNA levels that lead to inhibition of insulin secretion to an extent of 80% (19), such studies did not consider the oxidative stress that affects insulin metabolism in pancreatic islets. In the

present study, CsA caused further inhibition of calcineurin in such cells. The increased accumulation of thiobarbituric acid reactive substances and decreased activities of SOD indicate the ability of CsA to cause oxidative stress, a finding that is in agreement with recent reports made in this regard (20, 21). Furthermore, it was observed that culturing insulinoma cells in presence of CsA resulted in increased glutathione peroxidase activity to levels that were observed in normal pancreatic cells and may be responsible for combating increased oxidative stress seen in

response to CsA. The combined results of the present study point towards the role of calcineurin as a sensor of oxidative stress in order to mediate redox regulation of insulin secretion and synthesis.

ACKNOWLEDGEMENTS

Financial support to CS through research grants awarded by CSIR and DST are acknowledged. TKS acknowledges Chief executive BRIT, Mumbai and the Officer -in -Charge, JONAKI, CCMB, Hyderabad, for their support during the course of the work.

Table 1: Oxidative stress and calcineurin activity in normal islet cells and insulinoma cells.

	Pancreatic β -cells	Insulinoma cells	Insulinoma cells cultured in presence of CsA (3 μ M)
Calcineurin (Units)	31.4 $\pm$ 4.7	10.1 $\pm$ 0.6	5.6 $\pm$ 0.1*
Superoxide dismutase (Units)	2.1 $\pm$ 0.1	2.1 $\pm$ 0.1	1.1 $\pm$ 0.02*
Glutathione peroxidase (Units)	5.9 $\pm$ 0.3	3.8 $\pm$ 0.4	6.1 $\pm$ 0.5**
Reduced glutathione [GSH] (ng/mg protein)	4.0 $\pm$ 0.1	0.9 $\pm$ 0.1	0.7 $\pm$ 0.02**
Oxidized glutathione [GSSG] (ng/mg protein)	3.1 $\pm$ 0.04	3.1 $\pm$ 0.3	3.7 $\pm$ 0.1**
Thiobarbituric acid – reactive substances (nmoles/mg protein)	4.0 $\pm$ 0.1	6.7 $\pm$ 0.6	10.6 $\pm$ 0.7*

Mean $\pm$ S.D. values obtained from three separate experiments. p values (* = < 0.0001 and

** = < 0.01) are in relation to insulinoma cells cultured in absence of Cyclosporin A (CsA)

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