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Inflammation-induced ROS generation causes pancreatic cell death through modulation of Nrf2/NF-κB and SAPK/JNK pathway

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Abstract

Chronic pancreatitis is characterized by progressive loss of exocrine and endocrine functions of the pancreas and is considered to be the single most important cause for development of pancreatic cancer. Recent evidence suggests that inflammation and oxidative stress play pivotal roles in the development of clinical conditions like pancreatitis, type 2 diabetes mellitus, and metabolic syndrome. Nonetheless, molecular signaling pathways linking inflammation, oxidative stress, and pancreatic cell death are not yet well defined. In this study, bacterial lipopolysaccharide (LPS) was used (injected twice a week for three weeks) to emulate a chronic systemic inflammatory state in experimental Swiss albino mice. Using this model, we traced the genesis of inflammation-induced pancreatic dysfunction and mapped the signaling events which contribute to the induction of this state. Histopathological studies revealed the appearance of cell injuries and increased collagen content in LPS-exposed group, indicative of fibrosis. Assays for intraperitoneal glucose tolerance, insulin levels, and insulin receptor mRNA expression signified inflammation-induced insulin insensitivity. For the first time we present evidence that cellular oxidative stress possibly play important roles in the development of these clinical conditions [2,3]. Lifestyle factors like cigaret smoking, alcohol consumption, and inflammatory conditions of unknown origin are known to drive chronic inflammation [4]. In a state of chronic inflammation, the endocrine function of the pancreas is disrupted which culminates as glucose intolerance, pancreatitis, and T2DM [5]. Insulin resistance is a pathological condition arising out of impaired insulin signaling. One of the mechanisms leading to insulin resistance is low-grade inflammation which involves, among other factors, reactive oxygen species (ROS) [6].

Oxidative stress is essentially a dysregulation of the redox balance of the cellular environment, which under homeostatic conditions is reducing in nature [7]. Inflammation may be identified as the biological response to oxidative stress, which initiates the restoration of homeostasis [8]. From the perspective of development of metabolic disorders, inflammation and oxidative stress are very closely related [9]. The transcription factor nuclear factor (NF)-E2-related factor 2 (Nrf2) represents a crucial protection system in higher organisms, as it drives ROS detoxification [10] through the induction of expression of many antioxidant enzymes, such as superoxide dismutase (SOD) and heme oxygenase (HO)-1. NF kappa-light-chain-enhancer of activated B cells (NF-κB) is a key transcription factor that regulates cellular responses to oxidative stress by coordinating a pro-inflammatory response [10]. When NF-κB-mediated attempts to restore homeostasis fail and oxidative stress rise to extreme levels, cellular apoptosis is triggered [11].

Oxidative stress is widely observed to activate the stress kinases p38 and stress-activated protein kinase (SAPK)/Jun-N-terminal kinase (JNK) pathways, leading to activation of stress-response genes [12]. Depletion of cellular antioxidant defense allows for the generation of significant quantities of ROS, which has been suggested to induce
apoptosis [13]. In an interesting study, melatonin, a known antioxidant, was shown to prevent cerulein-induced pancreatic injury by modulating oxidative stress via the Nrf2 pathway in parallel with reduction of inflammation by NF-κB inhibition [14].

The precise molecular signaling networks that link inflammation, oxidative stress, and pancreatic cell death are not defined. In our study, we have established that inflammation-associated ROS plays a pivotal role in pancreatic dysfunctioning. To create a chronic inflammatory profile in experimental mice, bacterial lipopolysaccharide (LPS) was used. LPS is reported to activate the immune system through toll-like receptor-4 (TLR-4) receptor [15] and upregulate pro-inflammatory cytokine production [16]. Immune cells express certain pattern recognition receptors (PRRs) which recognize immune stimulatory microbial products. This family of PRRs includes TLR-4 family. TLR-4s are present on immune cells like macrophage and monocytes. Activation of TLR-4 receptor leads to secretion of several immune regulatory molecules. LPS is able to activate TLR-4 receptor and trigger inflammatory pathways [17]. LPS signaling has been reported to be responsible for NF-κB activation. Inhibition of TLR-4 binding to LPS could decrease NF-κB activation, inhibit inflammatory response, and prevent inflammatory carcinogenesis [18]. Using our model, we have traced the signaling events which induce chronic inflammation-mediated pancreatic dysfunction, which in turn result in insulin insensitivity and pancreatic cell death. In this study for the first time we present evidence that cellular oxidative stress, as induced by LPS, modulates NF-κB/Nrf2 signaling, triggers the mitogen-activated protein kinase (MAPK) stress signaling, and induces Rho/SAPK/JNK-dependent cell death. JNK is crucially implicated in the onset of insulin resistance and its inhibition improves insulin sensitivity in obese mice without affecting body weight and food uptake [19]. The data obtained led us to surmise that the LPS/TLR-4/ROS/NF-κB pathway plays a pivotal role in the initiation of oxidative stress and inflammation and it might prove to be an excellent choice as a target for prevention of inflammatory diseases.

Materials and methods

Materials

All the reagents were of analytical grade and were purchased from SRL India and Sigma Aldrich, USA. Anti-Rho-a, anti-SAPK/JNK, anti-TLR-4, anti-Nrf2, anti-p53, anti-beta-actin, and anti-H2B antibodies were purchased from Cell Signaling Technology, Inc. USA. Anti-p-phosphoinositide-3-kinase (PI3k) and anti-protein kinase B (AKT), anti-cyclooxygenase 2 (COX2), anti-inducible nitric oxide synthase (iNOS), and anti-Bcl2 were purchased from Abcam (USA). Anti-Bax was purchased from BD Pharmingen. The secondary antibodies, both anti-mouse and anti-rabbit, were purchased from Santa Cruz biotechnologies (CA, USA).

Experimental animals

Animal maintenance and experimentation

Male Swiss albino mice, weighing-20 g (8 weeks old, purchased from registered animal breeders, Kolkata) were acclimatized under laboratory conditions for 1 week prior to experiment. Food and water was given ad libitum and animals were maintained at 12-h light and dark cycle [20]. All animal experiments were done according to the guidelines of the Institutional Animal Ethical Committee (IAEC), Department of Physiology, University of Calcutta, Kolkata. The study was approved by Institutional Animal Ethics Committee or IAEC and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi, India (permit number: 820/04/ac/CPCSEA). The animals (n = 6 for each group) were divided into (i) control and (ii) LPS-exposed group.

LPS exposure

The experimental animals were exposed to LPS (i.p) at the dose of 100 µg/kg body weight (100 endotoxin units) of LPS exposed on day 1 and day 4 of a week for three consecutive weeks [21].

Pancreatic cell isolation

Pancreas was isolated from the control and experimental animals after anesthesia and cervical dislocation in a sterile condition, following the method of Aspinwall et al. 1998 [22] with minor modifications. Collagenase P (0.8 mg/ml) was injected into the bile duct and the engorged pancreas was isolated gently and all the other tissues were removed. The pancreas was then incubated in ethylenediaminetetraacetic acid (EDTA) (0.05 M) for 15 min and then dispersed into single cells by gently shaking in phosphate-buffered saline (PBS). Cells were cultured at 37°C, 5% CO₂ in RPMI (pH 7.4) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Cytotoxicity markers

Serum amylase and lactate dehydrogenase (LDH) activities were measured using commercially available kits from Span Diagnostics, India. The LDH kit is linear up to 2000 IU/L. To achieve linearity using the serum amylase assay kit, the absorbance of control sets at 620 nm should read 0.6 ± 0.05. Our experimental controls showed similar absorbance at 620 nm. All experiments were repeated thrice to confirm specificity and sensitivity.

Intraperitoneal glucose tolerance test (IPGTT)

Control and experimental animals were fasted overnight (12 h) before administration of dextrose (Sigma Aldrich, USA) dissolved in PBS via intraperitoneal (i.p) injection (2 g/kg body weight). Whole blood was once collected from
the tail vein before i.p glucose administration. The blood glucose level was measured by automated glucose level detector (Bayer Pharmaceuticals Pvt. Ltd.). After administering dextrose into the peritoneal cavity, blood was collected from the tail vein at intervals of 15, 30, 60, 90, and 120 min. After intraperitoneal glucose tolerance test (IPGTT), the animals were sacrificed and pancreas was collected [23].

Insulin production and cytokine assay

Blood from retro-orbital plexus of mice was collected from the experimental groups. Collected blood was kept at 4°C for 4 h in a slant position. It was cold centrifuged at 37 g for 10 min. Clear serum was collected from above the clot. 75 μl of serum was loaded in the enzyme-linked immunosorbent assay (ELISA) plates. Serum insulin, and tumor necrosis factor alpha (TNFα), interleukin (IL)-6, and IL-10 cytokine levels were measured using RayBio-tech ELISA Kits. According to the manufacturer’s manual, the expected linearity range is 94% for the cytokines and 78% for insulin. The reproducibility of both inter- and intra-assay is coefficient of variation or CV < 10% for cytokines and < 12% for insulin. All experiments were repeated thrice to confirm specificity and sensitivity.

Histopathological studies

Hematoxylin and Eosin (H&E). For histological studies, the pancreatic tissues collected from all the groups were fixed with 10% phosphate-buffered neutral formalin, dehydrated in graded (50–100%) alcohol, and embedded in paraffin. Thin tissue sections (4–5 μM) were cut and stained with routine hematoxylin and eosin (H&E) stain for photomicroscopic assessment

Picrosirius Red staining. To estimate the collagen content of pancreas, Picrosirius Red (SKL, India) staining was done. The tissue sections were deparaffinized in xylene and were hydrated using a series of washes in decreasing grades of alcohol. Adequate Picrosirius Red stain was applied to cover the tissue and was incubated at room temperature for 60 min. The tissue sections were washed in acidified water, dehydrated, and mounted in mounting medium [24].

Nitric oxide (NO) release

Determination of NO release was done using the serum of experimental animals. 0.5 ml of serum from control and LPS group was mixed with 0.5 ml of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamidehydrochloride in 3% H₂PO₄ mixed in equal proportion). The absorbance was immediately measured at 550 nm. The amount of nitrite was calculated from standard curve obtained by different concentrations of sodium nitrite [25].

Preparation of cytosolic and nuclear lysate for determination of nuclear localization of Nrf2 and NF-κB

Isolated pancreatic cells were suspended in ice-cold buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH: 7.9, 2 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) with freshly added protease inhibitor cocktail (Sigma Aldrich). After sonication, the samples were left on ice and cold centrifuged at 25,000 g for 10 mins. The supernatant was collected as cytosolic lysate and was stored at −80°C. The pellet was re-suspended in a buffer containing 10 mM HEPES (pH: 7.9), 300 mM NaCl, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF, and 10% glycerol with freshly added protease inhibitor cocktail. The samples were kept at 4°C overnight and then cold centrifuged at 25,000 g for 10 mins. The suspended extract was stored in −80°C as nuclear lysate. Protein estimation of the samples was done according to Lowry’s method. [26]

Western blotting

Cell lysates for Western blotting was prepared following Mukherjee et al. 2013 [20]. Equal amount of cell lysate were subjected to immunoblotting. The monoclonal antibodies used were anti-COX-2, anti-iNOS, anti-Rho-a, anti-TLR-4, anti-SAPK/JNK, anti-mouse Beta-actin, anti-Bax, anti-Bcl2, anti-caspase-3, anti-PI3K, anti-p-IκB, anti-p65, and anti-Nrf2. The secondary antibodies were alkaline-phosphatase-conjugated goat anti-rabbit antibodies. The positive bands were visualized using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate or NBT/BCIP (1:1) developing buffer. In parallel experiments, equivalent amount of protein was Western blotted with appropriate antibodies to confirm equal loading. Beta-actin was used as the loading control. Densitometry was done using Image J software.

Fluorescence microscopy

Pancreatic cells (single-cell suspension) were cold centrifuged at 11,400 g (for 5 min) and then washed twice in PBS. The cells were then fixed in 1% paraformaldehyde, washed with PBS, and then blocked with 5% bovine serum albumin (Sigma Aldrich) for 1 h at 4°C and then washed thrice in PBS. The cells were re-suspended in PBS containing (1:1000 diluted) fluorescence-tagged primary antibody specific for mouse p53 and p65 (BD PharmigenTM) and incubated in dark at room temperature for 30 min. Essentially for nuclear translocation assays the cells were washed twice with PBS post primary antibody staining and then counterstained with 1 mM 4′,6-diamidino-2-phenylindole (DAPI) in PBS and kept at room temperature for 45 min and washed twice with PBS and then visualized under fluorescence microscope (BD Pathway). The images were analyzed and edited for appropriate resolution using ImageJ software [27].

Flow cytometry for detection of intracellular ROS generation

Isolated pancreatic cells collected from animals were washed three times with chilled PBS and then exposed to 15 μM of dihydroethidium (DHE) and incubated for 20 min.
Excess DHE was washed off from the cells to avoid absorption of any extracellular oxyethidium formed by auto-oxidation of dihydroethidium. The cells were re-suspended in PBS and the fluorescence intensity was measured by BD FACSVerse flow cytometer and the data was analyzed using FlowJo software (Version 7.6.5) [28].

**Preparation of post-mitochondrial supernatant**

Pancreas were isolated from the control and experimental animals and homogenized in chilled PBS with KCl (1.17% w/v). The homogenate was centrifuged at 800 g for 5 min at 4°C, to separate the nuclear debris. The supernatant was centrifuged at 10,500 g for 20 min to obtain the post-mitochondrial fraction [29].

**Estimation of lipid peroxidation (LPO)**

Level of lipid peroxidation in post-mitochondrial supernatant was assayed following the method of Wright et al 1981 [30]. Malonaldehyde or MDA which is the end product of LPO reacts with thiobarbituric acid (TBA) to form a colored compound which is measured spectrophotometrically at 535 nm and calculated using a molar extinction coefficient of 1.56 x 10^5 M/cm.

**Superoxide dismutase activity**

Superoxide dismutase (SOD) activity was assayed following the method of Marklund et al. 1985 [31] based on the auto-oxidation rate of pyrogallol at 420 nm and inhibition of this auto-oxidation by SOD present in post-mitochondrial supernatant, where 50% inhibition corresponded to one unit of enzyme activity. SOD activity was expressed in units/mg of protein.

**Catalase (CAT) activity**

Catalase (CAT) activity was assayed following the method of Claiborne et al. 1985 [32]. The decomposition of catalase was measured spectrophotometrically at 240 nm. The assay is based on the decomposition of H_2O_2 to H_2O and O_2; by catalase present in the pancreatic post-mitochondrial supernatant, CAT activity was expressed in terms of nmol H_2O_2 consumed/min/mg protein.

**Estimation of reduced glutathione (GSH) content**

Reduced glutathione (GSH) content in the PMS was assayed spectrophotometrically following the method of Jollow et al. 1974 [33]. The sulfhydryl group of GSH reacts with 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and produces a yellow-colored TNB. The yellow color developed was measured at 412 nm. GSH content was measured from standard curve and expressed in μmoles/g of tissue.

**Determination of Heme oxygenase-1 (HO-1) and NAD(P)H Quinone Oxidoreductase 1 (NQO1) activity**

Heme oxygenase-1 (HO-1) activity was measured using the method of Carraway et al 2000 [34]. HO-1 activity was assessed by monitoring the biliverdin reduction to bilirubin by biliverdin reductase by measuring the differences in optical density at 464 nm and 530 nm with extinction coefficient of 40 mM/cm and the enzymatic activity expressed as pmole/h/mg of protein. NAD(P)H quinone oxidoreductase 1 (NQO1) activity was measured as the dicumarol-inhibitable fraction of 2,6-dichlorophenolindophenol (DCPIP) reduction according the method of De Haan et al 2002 [35]. The optical density was measured at 600 nm at room temperature at an interval of 60 s. The DCPIP reduction was expressed as reduced/min/μg of protein.

**Cell viability assay**

Pancreatic cells were harvested from mice and were cultured under different doses of LPS (0, 0.5, 1.0, and 1.5 μg/ml). Viability of cells were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The cultured cells were re-suspended in 200 μl of PBS and incubated with 20 μl of MTT (5 mg/ml) for another 4 h. The medium was aspirated off and 200 μl of dimethyl sulfoxide (DMSO) was added to each group. The formazan salt was dissolved in DMSO and absorbance read spectrophotometrically at 540 nm. N-acetyl-L-cysteine (NAC) (10 mM) was used as an experimental control. Cell viability was measured as a percentage of the control culture [36].

**Trypan blue assay**

Trypan blue dye exclusion test was used to measure the pancreatic cell viability. 50 μl of pancreatic cell suspension was mixed with equal volume of 0.4% Trypan blue solution (Sigma Aldrich). 10 μl of the stained cells was then loaded in the hemocytometer and examined immediately under light microscope at 40 × magnification. The total number of cells and the cells with blue stain were counted. Calculation for percentage viability = stained cell count/total cell count* 100 [37].

**Total RNA isolation and PCR analysis**

Pancreatic cells (1 x 10^6 cells/ml) were harvested and used for total RNA extraction by TRIzol reagent (Ambion RNA by Life Technologies). 200 ng of total RNA was reverse transcribed to cDNA using Maxima H Minus Reverse Transcriptase (2000 U; Thermo SCIENTIFIC) and polymerase chain reaction (PCR) amplification was done by premix kit containing DNA polymerase, dNTPs, MgCl₂, and buffer (Life Technologies); PCR amplification conditions were 1 cycle of initial denaturation (95°C for 60 s) and 35 cycles of amplification (95°C for 15 s, 59°C for 15 s, and 72°C for 3 min). Primer for insulin receptor gene was designed and synthesized using primer 3 software. The primers used for analysis are as follows: insulin receptor (IR) Forward 5′-AAAGTGTTGCCCAACCATCCTG-3′ Reverse 5′-GTTAAGCTTGCAGAAGC-3′, Beta-Actin Forward 5′ TTCTTACACTGCGGACGACA-3′ Reverse 5′-GGGGTGTGAAAGGTCTCAA-3′. The relative expression of a given mRNA was assessed by
normalizing the value with beta-actin and comparing with normal value. All primer sets were tested prior to use in this work to ensure that only a single product of the correct size was amplified [38].

Statistical analysis

All values were expressed as mean ± standard error of mean (SEM). Significant differences between the groups were determined with GraphPad Prism 6 software for Windows using one-way analysis of variance or ANOVA and the group means were compared using two-tailed Student’s t-test, and P values lower than 0.05 (p < 0.05) were considered significant.

Results

LPS induces cytotoxicity leading to insulin insensitivity in pancreatic cells

In order to assess pancreatic cell damage due to sustained low-grade inflammation in mice, amylase and LDH activities were determined from the serum of animals in both the control and LPS-administered groups. It was observed that both the serum amylase and LDH activities were significantly higher (p < 0.05) with respect to control values in the LPS-treated group (Figure 1A and B). The amylase activity was about 2.5 fold higher and the LDH activity was 6 fold higher in the LPS-treated group with respect to control (Figure 1A and B). It was observed in

Figure 1. Sustained low-grade inflammation results in cellular toxicity and insulin insensitivity in pancreas. (A), (B) Serum amylase and LDH activities in control and LPS-administered Swiss albino mice (100 μg/kg LPS i.p; twice weekly for three consecutive weeks). (C), (D) Serum glucose and insulin concentration in control and LPS-administered animals after IPGTT. (E) IR mRNA expression in pancreas for the control and LPS groups after each week. All values are mean ± SEM, * indicates significant change with respect to control at p < 0.05 and p < 0.01, respectively.
our experiments that LPS-administered mice showed significant decrease ($p < 0.05$) in serum insulin levels with respect to control (Figure 1D). To ascertain hyperglycemic condition in the LPS-administered animals, we performed IPGTT. Rise in glucose level both in control and LPS-treated group was observed after 15–30 min. The level gradually decreased after 60 min in control group but no significant decrease in glucose level was observed in the LPS-treated group. At every point of time the glucose level in the LPS group was significantly higher ($p < 0.05$) than control (Figure 1C).

To evaluate the possible involvement of insulin in the alteration of the glucose metabolism, we estimated the serum insulin levels after IPGTT at each time point. It was very interesting to observe that the insulin level after 60 and 90 min of glucose injection (i.p) in the LPS-administered animals was significantly decreased ($p < 0.05$) with respect to the control group (Figure 1D).

Pancreatic dysfunction may also potentiate deregulated IR gene expression or signaling, thereby hampering glucose metabolism through the downregulation of insulin receptor expression, we evaluated the mRNA expression of IR with respect to the control group (Figure 1E) by PCR at the end of each week of LPS administration. It was observed that there was a significant decrease ($p < 0.05$) in IR expression at the end of 3rd week of LPS administration (Figure 1E). This decreased expression of IR gives a vivid picture of inflammation-induced pancreatic dysfunction and related alterations in insulin-dependent glucose metabolism. These results together clearly indicate that a sustained inflammatory state, as induced by LPS, causes pancreatic dysfunction and insulin insensitivity.

**LPS induces degenerative changes in the pancreas along with increased lymphocyte infiltration**

Deparaffinized slides of pancreas were H&E stained and then visualized under bright-field microscope at 10 and 40X, respectively. Pro-apoptotic changes were observed in the LPS-treated group as evidenced by increased number of pyknotic nuclei (Figure 2A). Pancreatic islets were also sparse (Figure 2D) in the LPS-administered group. Atrophy of the pancreatic acinar cells was observed (Figure 2C). Increased lymphocyte infiltration in the pancreas of the LPS-treated group suggests involvement of the immune system in the onset of pancreatic inflammation (Figure 2A). Control animals did not show any of the above alterations. These results establish that sustained low-grade inflammation could induce pancreatic malfunctioning.

Picrosirius staining (Figure 2B) of the pancreatic tissues revealed an increase in collagen content in the LPS-treated group compared with that in the control pancreas. Damaged areas become positive for collagen in the pancreatic tissues of the LPS-treated mice. Normal cellular architecture and basal level of collagen are observed in the control group (Figure 2B).
LPS potentiates inflammatory responses in pancreas through the TLR-4/NF-κB pathway

Immune expression of TLR-4 in both in vivo (Figure 3E) and in vitro (Figure 6A) study showed increased expression of TLR-4 receptor. Dose-dependent activation of the receptor was observed in the in vitro study (Figure 6A). In our experiments it was observed that there was a significant rise ($p < 0.05$) in the serum nitric oxide (NO) level, and IL-6 and TNF-α levels (Figure 3A, B and C) in the LPS-administered group with respect to control along with noticeable decrease in IL-10 values (Figure 3D) and these results were accompanied with significant increase ($p < 0.05$) in the phosphorylation of the downstream mediators of TLR-4 pathway. Phosphorylation of AKT, PI3K, and IκB (Figures 3E and 6A) was observed to be increased in both in vivo and in vitro study with increased nuclear localization of NF-κB (p65) (Figures 3E and 6B).

To confirm p65 nuclear localization, confocal imaging was performed which corroborated that there was increased nuclear localization of the p65 protein in the LPS group (Figure 3F). The expression of downstream mediators of the TLR-4/NF-κB pathway, COX-2 and iNOS, was evaluated and it was observed that there was an increased expression of both these proteins in the pancreas of the LPS-exposed animals (Figure 3G), which implicates that LPS induces inflammation in the pancreas through the activation of the TLR-4 pathway.

Inflammation-induced ROS generation critically alters cellular redox homeostasis in the pancreas

To explore the role of sustained low-grade inflammation as induced by LPS in generating oxidative insult to the pancreatic cells, we estimated intracellular ROS levels and it was observed that LPS-administered animals showed increased ROS generation within the pancreatic cells with respect to control (Figure 4A). In our previous work we have established that disturbed redox condition in cells abrogates Nrf2-dependent antioxidant defense, thereby influencing the cells to progress toward initiating death programs [20].

In order to understand the status of the Nrf2-dependent pathways in LPS-exposed pancreatic cells, we evaluated the nuclear localization of Nrf2 and found that there was decreased nuclear translocation of Nrf2 in the pancreatic cells of LPS-inflamed animals (Figure 4B), suggesting a probable deregulation of the antioxidant defense mechanism within the pancreas.

In order to validate the redox status, we evaluated the SOD, CAT, HO-1, and NQO-1 activities followed by...
TBA-reactive substances (TBARS; LPO) and GSH level in the pancreas. It was observed that there was a significant decrease ($p < 0.05$) in the activities of SOD, CAT, HO-1, NQO-1, and the GSH level (Figure 4D, E, F, G and H) with concomitant increase in the LPO level (Figure 4C) in the pancreas after three weeks of LPS administration. All values are mean ± SEM. * indicates significant change with respect to control at $p < 0.05$ and $p < 0.01$, respectively.

**Systemic low-grade inflammation induces intrinsic cell death pathways in pancreas**

In one of our previous works we have established that increased ROS generation induced by altered cellular redox homeostasis could induce the SAPK/JNK-initiated apoptotic pathway [20]. It was observed upon DAPI staining that there were increased numbers of fragmented nuclei in the LPS-administered group with respect to control (Figure 5A).

We further investigated the probable pathway involved in inflammation-induced death of pancreatic cells. It was observed in our experiment that there was a significant increase ($p < 0.05$) in the expression of the small GTPase family protein Rho-a, with accompanying increase in the expression of pro-apoptotic proteins like Bax and caspase-3, increased phosphorylation of SAPK/JNK (Figure 5C), and increased nuclear translocation of p53 (Figure 5B) along with significant decrease ($p < 0.05$) in the expression of the anti-apoptotic protein Bcl2 (Figure 5C), indicating the induction of SAPK/JNK-mediated apoptotic pathway within the cells (Figure 5C).

**Inflammation-induced ROS orchestrate the modulation of NF-κB-Nrf2 and activation of SAPK/JNK-mediated intrinsic death pathway in pancreatic cells: validation of the hypothesis**

To validate our hypothesis that persistent increase in intracellular ROS due to sustained inflammation may lead to pancreatic dysfunction and cell death, we simulated the in vivo inflammatory condition by culturing primary pancreatic cells isolated from Swiss albino mice with increasing doses of LPS (0.5, 0.1, and 1.5 μg/ml). We intended to establish whether LPS could potentiate the activation of TLR-4 pathway within the pancreatic cells in vitro. It was observed that with increasing doses of LPS there was a significant increase ($p < 0.05$) in the phosphorylation of AKT, PI3K, and I-κB along with increased nuclear translocation of p65, with respect to control (Figure 6A).

Similar results were noticed in the in vivo experiments. The results obtained strongly implicate that there is indeed activation of the intracellular TLR-4 inflammatory pathways in these cells.

As ROS is a critical signaling intermediate in inflammatory conditions, we needed to validate whether ROS is the crucial link between inflammation and induction of apoptosis within the LPS-exposed cells. The expression of
Rho-a, Bax, caspase-3, and Bcl2, and the phosphorylation status of SAPK/JNK proteins were evaluated. It was observed in our experiments that with increasing doses of LPS there was a significant increase \( p < 0.05 \) in the expression of Rho-a, Bax, and caspase-3 and the phosphorylation of SAPK/JNK with significant decrease \( p < 0.05 \).

Figure 5. Inflammation-induced oxidative insult in pancreas activates the intrinsic pro-apoptotic pathway. (A) Nuclear staining by DAPI shows increased nuclear fragmentation in the LPS group as indicated by “yellow arrows”. (B) p53 nuclear translocation in pancreas. (C) Representative Western blots for expression of different proteins in the SAPK/JNK-mediated cell death pathway in pancreas. The right-hand panels represent densitometric analyses of the blots. All values are mean ± SEM, *, ** indicates significant change with respect to control at \( p < 0.05 \) and \( p < 0.01 \), respectively.

Figure 6. ROS is a critical factor for the activation of SAPK/JNK-mediated cell death pathway in pancreas. (A) Representative Western blot for the expression of TLR-4-mediated inflammatory pathway proteins in pancreas in vitro after 0.5, 1.0, and 1.5 \( \mu \)g/ml of LPS administration for 24 h. (B) Demonstration of the expression of p65 nuclear localization with increasing dose of LPS for in vitro study. The panel below Figure 6B represents the densitometric analysis of p65. All values are Mean ± SEM, *, ** indicates significant change with respect to control at \( p < 0.05 \) and \( p < 0.01 \), respectively, # shows significant change with respect to LPS group at \( p < 0.05 \).
in the expression of Bcl2 suggesting toward the induction of the SAPK/JNK-mediated apoptotic pathway within the cells (Figure 7A).

MTT assay was performed using NAC as a negative control to pinpoint the involvement of ROS in inflammation-induced pancreatic cell death. It was observed that NAC could significantly reduce \( p < 0.05 \) LPS-induced pancreatic cell death as evidenced by decrease in the percentage of metabolically inactive cells after NAC supplementation to the LPS groups (Figure 7B and C). Trypan blue exclusion test was also performed to confirm cell death. Percentage of viable cells were less with increase in dose of LPS and the reverse was observed in the NAC-supplemented LPS groups (Figure 7D).

**Discussion**

Pancreatitis is a disease characterized by progressive destruction of pancreatic tissue, and as the disease evolves, significant impairment of exocrine as well as endocrine functions become evident. Chronic pancreatitis has been projected as a strong risk factor for pancreatic cancer. A meta-analysis study has shown a relative risk of 13.3 for developing pancreatic cancer among pancreatitis patients [40]. The pathophysiology of pancreatitis remains incompletely understood and so far it is associated with limited therapeutic options and poor prognosis. In this study we have traced the signaling pathways that play intricate role in developing inflammation-induced pancreatitis in an attempt to understand the underlying molecular governances.

Lipopolysaccharide is a structural component of Gram-negative bacteria. It is widely used to induce systemic inflammation. LPS binds with TLR-4 receptor and activates c-JNK which results in production of inflammatory cytokines like IL-1\( \beta \), IL-6, and TNF-\( \alpha \) [18]. We have administered LPS (twice a week) in mice for three consecutive weeks to stimulate the inflammatory pathways. At the end of the experiment it was observed that the serum amylase and LDH levels were significantly higher. Elevated levels of serum amylase and LDH are markers of chronic pancreatitis [41]. In addition to this observation, our histological data indicated pancreatic tissue injury. The red staining of Picrosirius Red reflects the occurrence of fibrosis and the increased bluish dots of hematoxylin-stained cells reflect nuclear condensation of the pancreatic cells. These observations give a qualitative confirmation of our hypothesis that LPS-induced inflammation induces pancreatic tissue injury.

Toll-like receptor-4 (TLR-4), belonging to a family of pathogen-associated molecular-pattern-recognition molecules, plays a key role in the initiation and acceleration of inflammation. Upon LPS exposure, TLR-4 undergoes oligomerization and recruits its downstream mediator through interaction with toll IL-1 receptor (TIR) domain. The NF-\( \kappa \)B signaling pathway, being downstream of TLR-4-mediated signaling, plays a critical role in amplifying inflammatory responses by upregulating the expression of various pro-inflammatory genes [42]. In our study increased phosphorylation of the upstream mediators of the NF-\( \kappa \)B pathway—AKT, PI3K, and IkB and downstream targets like COX-2 and iNOS confirms the LPS/TLR-4/NF-\( \kappa \)B pathway activation.

Activation of inflammatory pathways is positively associated with the generation of ROS. We have established in our previous work that NF-\( \kappa \)B activity is modulated by increased intracellular ROS produced due to inflammation.
Inflammation-induced pancreatic dysfunction and death

Significant elevation of TNF-α, accordingly Rho-a expression was analyzed to correlate inflammation with NF-κB activation. SAPK/JNKs are known as stress-activated kinases, and are responsive to a variety of exogenous and endogenous stress-inducing stimuli, including oxidative stress and pro-inflammatory cytokines. JNK/SAPK are activated by hyperglycemia-induced oxidative stress and are likely involved in apoptosis mediated by hyperglycemia in human endothelial cell [54].

Harijith and et al. (2014) [55] reviewed that oxidant damage from ROS plays a significant role in initiating cellular apoptotic processes. Our study revealed that the survival pathways of the pancreatic cells were arrested and apoptosis was initiated. Increased expression of pro-apoptotic Bax and decreased expression of anti-apoptotic Bcl2 provides an indication of the activation of apoptotic pathway. DAPI staining of pancreatic cells showed DNA fragmentation in the LPS-administered group. DNA fragmentation might possibly be associated with accumulation of free radicals within the pancreatic cells. DNA fragmentation is known to lead to the activation and nuclear localization of p53, which in turn activates SAPK/JNK-mediated apoptosis [56]. Nishina H et al. 2004 [57] have also recounted SAPK/JNK activation triggering mitochondria-dependent apoptosis in response to various kinds of cellular stress. Also the findings of Chen X et al. 1996 [58] report that DNA damage may trigger p53-mediated growth arrest and apoptosis. In our study, increased DNA fragmentation and expression of p-SAPK/JNK in the LPS group agrees with these findings of Enomoto A et al. 2001[59] and Chen X et al. 1996 [58]. Findings of our study also showed increased expression of caspase-3, the final executor of apoptosis. Altogether our data suggest that inflammation potentiates the activation of NF-κB/Nrf2 activation due to ROS accumulation which enhanced the inflammatory mediators, leading to pancreatic cell death by activation of p53-mediated activation of SAPK/JNK pathway of apoptosis.

To ascertain that sustained inflammation-induced generation of ROS is a critical mediator of the pro-apoptotic changes in pancreatic cells, we cultured isolated primary pancreatic cells with LPS in presence of a standard antioxidant molecule NAC. It was extremely interesting to note that there was decreased cell death in the presence of NAC (Figure 7B, C and D). This establishes the decisive role of ROS in mediating these pro-apoptotic changes in the cultured pancreatic cells. Our data conclusively identifies a lucrative target of possible intervention by potent antioxidants in mitigating inflammation-induced degenerative changes in the pancreas.

Conclusion

Together, the data presented endorse our hypothesis that an inflammatory condition is able to trigger ROS generation within the pancreatic cells thereby engaging the TLR pathway, leading to the increased production of inflammatory cytokines and nuclear localization of NF-κB. For the
first time our study established the role of SAPK/JNK in pancreatic dysfunction leading to the progression to insulin insensitivity and type-2 diabetes. Our findings also provide an insight that by modulating the activation of NF-kB and Nrf2, inflammation-induced pancreatic cell death could be reversed. Our study provides an option of intervening in the signaling pathways related to oxidative stress to eliminate the consequences of sustained inflammation, caused due to lifestyle disorders.

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Declaration of interest

The authors declare that no conflict of interest exists. The authors alone are responsible for the content and writing of the paper.

References

Inflammation-induced pancreatic dysfunction and death


Reactive oxygen species in the tumor niche triggers altered activation of macrophages and immunosuppression: Role of fluoxetine

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Keywords: Tumor Inflammation Macrophage polarization Immunosuppression Fluoxetine

Abstract

Macrophages are projected as one of the key players responsible for the progression of cancer. Classically activated (M1) macrophages are pro-inflammatory and have a central role in host defense, while alternatively activated (M2) macrophages are associated with immunosuppression. Macrophages residing at the site of neoplastic growth are alternately activated and are referred to as tumor-associated macrophages (TAMs). These “cooperate” with tumor tissue, promoting increased proliferation and immune escape. Selective serotonin reuptake inhibitors like fluoxetine have recently been reported to possess anti-inflammatory activity. We used fluoxetine to target tumor-associated inflammation and consequent alternate polarization of macrophages. We established that murine peritoneal macrophages progressed towards an altered activation state when exposed to cell-free tumor fluid, as evidenced by increased IL-6, IL-4 and IL-10 levels. These polarized macrophages showed significant pro-oxygen bias and increased p65 nuclear localization. It was further observed that these altered macrophages could induce oxidative insult and apoptosis in cultured mouse CD3+ T cells. To validate these findings, we replicated key experiments in vivo, and observed that there was increased serum IL-6, IL-4 and IL-10 in tumor-bearing animals, with increased % CD206+ cells within the tumor niche. TAMs showed increased nuclear localization of p65 with decreased Nrf2 expression in the nucleus. These results were associated with increase in apoptosis of CD3+ T cells co-cultured with TAM-spent media. We could establish that fluoxetine treatment could specifically re-educate the macrophages both in vitro and in vivo by skewing their phenotype such that immune suppression mediated by tumor-dictated macrophages was successfully mitigated.

1. Introduction

It has become increasingly apparent that inflammation plays a major role in tumor development and progression [1]. Multiple immunosuppressive mechanisms operate within the tumor microenvironment and confer resistance to therapeutic regimens. The tumor microenvironment is governed by pro-inflammatory conditions which is influenced by several cytokines like IL-6, IL1β, TNF-α or reactive oxygen/nitrogen species (ROS/RNS) released from immune cells present in the tumor vicinity [2]. Macrophages are considered as critical links in the connection between inflammation and cancer [3,4], they represent up to 50% of the tumor mass and produce a wide array of inflammatory mediators with pro-tumoral functions [3,4]. Furthermore, they have been associated with alterations in adaptive immune responses within the tumor microenvironment that may potentially lead to reduced immune surveillance [4,5]. The pro-inflammatory condition in the tumor microenvironment triggers the activation of naïve macrophages into an altered state [2], favoring tumor progression. These activated macrophages release soluble factors like cytokines or ROS/RNS that play critical role in immune alterations by inducing apoptosis in mononuclear cells, specifically lymphocytes [2,6,7]. The pro-inflammatory conditions in the tumor vicinity have been reported to activate pro-apoptotic cascades in thymocytes [8], and recent evidence also suggests that altered or activated macrophages induce oxidative damage in T cells through elevated expressions of pro-inflammatory cytokines, induction of iNOS and secretion of elevated levels of ROS/RNS [9].

The pivotal questions as to how tumor-associated inflammation induces macrophages to attain the altered state and the contribution of these altered macrophages to immune alterations are yet to be answered with clarity. Therefore, identifying strategies to alter the
Gold-conjugated green tea nanoparticles for enhanced anti-tumor activities and hepatoprotection — synthesis, characterization and in vitro evaluation

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Abstract

Green tea (GT)-based chemoprevention has shown promising results in various cancer models. However, the effective dose may not be far from the toxic dose because of inefficient systemic delivery and limited bio-availability of GT polyphenols. We have used GT polyphenols to successfully reduce gold to corresponding gold nanoparticles (NPs) in a single step; a process that fulfills all criteria of green nanotechnology as no “man-made” chemical other than gold acids are used. GT and (−) - epigallocatechin-3-gallate (EGCG) conjugated gold NPs (diameters <50 nm), showed remarkable stability, significantly rapid cellular uptake and excellent in vitro anti-oxidant activities. These NPs were observed to be selectively toxic towards cancer cells (Ehrlich’s Ascites Carcinoma and MCF-7) while showing absolutely no lethality towards normal primary mouse hepatocytes. In cancer cells, NPs altered the redox status and limited Nrf2 activation by almost 50%. These NPs significantly decreased nuclear translocation of NF-κB, coupled with decreased phosphorylation of IκB and down-regulation of NF-κB-dependent anti-apoptotic proteins Bcl2 and Akt in a dose-dependent manner, triggering onset of apoptosis. Culturing normal hepatocytes with tumor-conditioned media prompted apoptosis by increasing reactive oxygen species (ROS) and depleting the anti-oxidant defense mechanism of hepatocytes. Pretreatment with NPs protected hepatocytes from tumor-induced cellular damage by scavenging excess ROS, increasing the levels of reduced glutathione and anti-oxidant enzymes. There was evidence of decreased Bax/Bcl2 ratio and active Caspase 3 levels in these hepatocytes, indicating apoptosis escape. Nanoformulations of GT-based polyphenols might serve as an operative platform for effective delivery, increased bio-availability, enhanced effects and minimal chemotherapy-associated toxicities.

Keywords: Green tea; EGCG; Nanochemoprevention; Hepatotoxicity; Apoptosis; Nrf2; NF-κB

1. Introduction

Chemotherapy has long and routinely been used in the treatment of cancers. However, the use of conventional chemotherapy has been limited by accompanying dose-related toxicities, as well as the emergence of drug resistance. It is therefore of utmost importance to explore and establish novel drugs and delivery systems to minimize chemotherapy-induced toxicity while obtaining maximum anticancer efficacy [1].

Phytochemicals have aroused great interest as treatment options for cancers because of their minimal side effects [2]. Green tea (GT) is the most popular beverage produced from the leaves of Camellia sinensis. It contains various flavonoids; predominant among them are catechins, including epicatechin (EC), its hydroxyl derivative epigallocatechin (EGC) and their gallic acid esters, epicatechin-3-gallate (ECG) and epigallocatechin-3-gallate (EGCG) [3]. GT catechins have attracted attention as cancer-preventive agents due to their low toxicity and ready availability to the general population, as well as exerting preventive effects against cancers in humans [4]. Among these, EGCG is the most abundant and has been shown to exhibit anti-oxidative and chemo-preventive properties against various types of cancers because of its potent capacity for inhibiting cancer cell growth through several signaling pathways [5–7]. Epidemiological studies have revealed that GT consumption is inversely associated with the progression of prostate cancer and the risk of breast cancer recurrence [8,9]. Steele et al. have shown that GT activates detoxification enzymes, such as glutathione S-transferase and quinone reductase, thus protecting against carcinogenesis [10]. Studies have shown that tea and/or tea polyphenols may inhibit tumorigenesis at different organ sites, including skin, lung, oral cavity, esophagus, stomach, small intestine, colon, liver, pancreas, and mammary gland [11]. However, epidemiological and clinical studies on humans have been inconclusive and inconsistent due to variables such as differences in tea preparation and consumption, the methods of tea production, the bioavailability of tea compounds, genetic variation in how people
Pomegranate reverses methotrexate-induced oxidative stress and apoptosis in hepatocytes by modulating Nrf2-NF-κB pathways☆

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Abstract

The clinical efficacy of the widely used chemotherapeutic drug methotrexate (MTX) is limited due to its associated hepatotoxicity. Pomegranate polyphenols are of huge health benefits and known to possess remarkable antioxidant properties capable of protecting normal cells from various stimuli-induced oxidative stress and cell death. In this study, we explored the protective role of pomegranate fruit extract (PFE) in ameliorating MTX-induced hepatic damage. Male Swiss albino mice exposed to MTX (20 mg/kg body weight) exhibited distinct markers of toxicity such as increased activities of enzymes alanine transaminase, aspartate transaminase, lactate dehydrogenase and alkaline phosphatase and also increased oxidative stress in liver evidenced by increased ROS generation and lipid peroxidation. Decrease in reduced glutathione levels, superoxide dismutase, catalase, hepatic heme oxygenase 1 and NQO-1 activities were also observed. Tracing the signal transduction pathways, it was seen that MTX exposure significantly increased nuclear translocation of NF-κB coupled with increase in phosphorylated IκB and down-regulation of NF-kappaB-dependent antiapoptotic protein Bcl-2. Treatment with MTX increased the expression of the apoptotic enhancer Rho/Cdc42 as well as the phosphorylation of SAPK/JNK. A shift in the Bax/Bcl-2 ratio towards apoptosis and increase in the caspase 3 level was also evident. Administration of PFE for 7 consecutive days before and after MTX challenge suppressed MTX-induced cell death, mitigated the injurious effects of MTX and offered protection against apoptosis. PFE was shown to reduce ROS generation in hepatocytes by activating the Nrf2-ARE pathway and inhibiting NF-κB as a consequence of which the antioxidant defense mechanism in the liver was up-regulated, thereby conferring protection against MTX-induced hepatotoxicity and apoptosis.

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Keywords: Pomegranate; Methotrexate; Hepatotoxicity; Oxidative stress; Nrf2; NF-κB; Apoptosis

1. Introduction

Methotrexate (MTX) is a cytotoxic antifolate drug. It allosterically inhibits dihydrofolate reductase, an enzyme participating in tetrahydrofolate synthesis [1]. MTX is extensively used in the treatment of cancer, autoimmune and inflammatory diseases [2], ectopic pregnancy [3] and for the induction of medical abortions [4], but the efficacy of this drug is limited due to associated hepatotoxicities [5]. The liver plays a central role in the bio-transformation and clearing of drugs, and hence, it is greatly susceptible to toxicity induced by such agents. Since the toxic effects of MTX in the liver have been reported to be mainly due to increased oxidative stress as a result of generation of free radicals and reactive oxygen species [6,7], it is therefore hypothesized that these toxicities could be minimized by adjuvant therapy using antioxidants. Polyphenolics, widely distributed in botanicals with significant amounts in vegetables, fruits and beverages, form an integral part of diet and possess strong free radical scavenging and antioxidant properties [8]. Pomegranate (Punica granatum, Punicaceae) is an edible fruit cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia and the United States. Pomegranate is rich in polyphenolic antioxidants, which include tannins, ellagitannins, anthocyanins, catechins, flavonoids, punicalagins, gallic and ellagic acids [9]. The soluble polyphenol content in pomegranate juice is between 0.2 and 1.0%, depending on variety [10]. Extracts from different parts of pomegranate fruit such as juice [8], peel [11] and seed [12] have been reported to exhibit strong antioxidant