Original Research

Impaired NADPH oxidase activity in peripheral blood lymphocytes of galactosemia patients

Mazen Al-Essa[1], Gursev S Dhaunsi[1], Wafa'a Al-Qabandi[1] and Islam Khan[2]

[1]Department of Pediatrics, Faculty of Medicine, Kuwait University, Kuwait; [2]Department of Biochemistry, Faculty of Medicine, Kuwait University, 10002 Kuwait

Correponding author: Gursev S Dhaunsi. Email: dhaunsig@hsc.edu.kw

Abstract

Galactosemia is an autosomal recessive disorder with a wide range of clinical abnormalities. Cellular oxidative stress is considered as one of the pathogenic mechanisms of galactosemia. In this study, we examined the activity of NADPH oxidase (NOX), a major superoxide-generating enzyme system, in peripheral blood lymphocytes (PBL) from galactosemia patients. PBL were isolated from galactosemia patients and healthy control subjects and used for cell culture studies and biochemical assays. PBL were cultured in the presence or absence of galactose or galactose-1-phosphate (Gal-1-P), and enzyme activities and/or gene expression of NOX, catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured in the cell homogenates. PBL isolated from galactosemia patients showed significantly reduced ($P < 0.01$) activities of catalase and GPx; however SOD activity remained unaltered. Galactosemia patients were found to have significantly ($P < 0.01$) increased levels of malondialdehyde (MDA) in blood lymphocytes. Enzymatic activity of NOX was significantly ($P < 0.001$) reduced in galactosemia patients; however, Western blotting revealed that NOX-1 protein was not significantly altered. Interestingly, levels of NOX activity in lymphocytes isolated from galactosemia patients significantly increased but remained subnormal when cultured in galactose-deficient medium for two weeks, indicating a galactose-mediated inhibition of NOX. Lymphocytes isolated from control subjects were found to have significantly ($P < 0.01$) reduced NOX activity when cultured in the presence of galactose or Gal-1-P for two weeks. These results show that galactose-induced cellular oxidative stress is not NOX mediated. However, impairment of the NOX system might be responsible for some of the clinical complications in galactosemia patients.

Keywords: galactosemia, NADPH oxidase, lymphocytes

***Experimental Biology and Medicine* 2013; 238: 779–786. DOI: 10.1177/1535370213480692**

Introduction

Galactosemia is an inherited metabolic disorder that results from an aberrant galactose metabolism caused by profound impairment of galactose-1-phosphate uridyl transferase (GALT), galactokinase and galactose-4-epimerase. The clinical features of galactosemia have been reported to vary with gender and race, but more importantly the underlying enzyme defect due to specific gene mutations, and range from acute symptoms such as hypoglycaemia, vomiting and diarrhoea to chronic malfunction of multiple organ systems causing liver cirrhosis, cataracts, neurological defects and an increased risk of *Escherichia coli* (*E. coli*) infections.[1–3] Classical galactosemia due to deficiency of uridyl transferase is associated with abnormal accumulation of galactose and galactose-1-phosphate (Gal-1-P) in body tissues and fluids, whereas galactokinase deficiency often results in galactosemia and galactosuria. Development and progression of the disease in patients with classical galactosemia have been widely linked to abnormally high levels of galactose and Gal-1-P. Blood cells from galactosemic patients have been shown earlier to form two to three folds of Gal-1-P as compared with normal cells;[4] however, the expression and activity of GALT and the cellular levels of Gal-1-P in galactosemia patients have been reported to vary among different ethnic groups due to specific genetic mutations.[5] Various mechanisms including cellular oxidative stress have been suggested to underlie the pathogenesis of galactosemia-induced complications;[6,7] however, it is not clear whether galactose and/or Gal-1-P are involved in the development of cellular oxidative stress. The latter is caused by an imbalance in the production of reactive oxygen and nitrogen species and their degradation or neutralization by antioxidant cellular defence mechanisms involving antioxidant enzymes and non-enzymatic molecules.[8,9]

Catalase, superoxide dismutases (SODs) and glutathione peroxidase (GPx) are the key antioxidant enzymes that degrade reactive oxygen species hydrogen peroxide, superoxide anions and lipid peroxides, respectively, and their activities have been shown to be altered in galactosemia.[10] Among reactive nitrogen species, nitric oxide has been implicated in the pathogenesis of galactosemia where this gaseous free radical has been shown to modulate endothelial cell behaviour and contribute towards galactosemia-induced retinopathy.[11] Besides several other sources of free radicals during cellular metabolism, NADPH oxidase (NOX), a complex multicomponent enzyme system, is responsible for the production of highly reactive superoxide anions. NOX was originally reported to be localized in phagocytes that rapidly consume molecular oxygen to generate superoxide anion as a microbicidal tool against infections. Besides its localization in phagocytes, NOX is now known to be present in several cell types and tissues with tissue-specific NOX homologues for heart, liver, brain kidney, blood lymphocytes and blood vessels, and NOX-mediated superoxide production has been reported to be vital for some physiological functions such as smooth muscle cell tone, vascular motility and cellular defence against invading infectious agents.[12,13] Activation of vascular NOX has been widely reported to play a role in the pathogenesis of cancer, atherosclerosis, hypertension and diabetes through superoxide anion-mediated oxidative stress. Impairment of phagocytic NOX activity, due to improper function of one or more of its components – p22phox, p47phox and p67phox – has been implicated in the susceptibility of congenital granulomatous disease patients to microbial infections.[14,15] Furthermore, galactose has been reported to impair bactericidal activity of leucocytes *in vitro*.[16,17] In view of these published reports, it can be speculated that NOX might have a role in the pathogenesis of classical galactosemia through regulation of cellular oxidative stress. Also, commonly reported bacterial (*E. coli*) infections in galactosemic patients can be possibly associated with the status of cellular NOX systems. Therefore, in this study, we have examined the levels of antioxidant enzymes, catalase, GPx and SOD, and enzymatic activity of NOX in peripheral blood lymphocytes (PBL) of galactosemia patients to assess the possible role of NOX-mediated superoxide anion production in pathogenesis of galactosemic conditions.

Patients and methods

Subjects

Blood samples were collected from 15 galactosemia patients and 15 healthy (age and sex matched) controls after obtaining informed consent. This study was carried out according to the protocols of the institutional ethical review committee of Kuwait University. Recruitment of galactosemic patients was based on confirmed deficiency of GALT enzyme (residual GALT-activity less than 5%) in erythrocytes of all patients. GALT-genotyping was not performed due to lack of resources. All the patients and controls were drug free and ranged from 6 months to 5.5 years of age. Although all patients were advised a lactose-free diet, non-compliance to diet modification was commonly observed, and this was supported by markedly elevated levels of liver enzymes (alanine transaminase, 236 ± 103 IU/L and gamma-glutamyl transferase, 96 ± 37 IU/L) in sera of galactosemia patients.

Materials

Chemicals and materials required for cell cultures were procured from Invitrogen (Carlsbad, CA, USA). Galactose, Gal-1-P, hydrogen peroxide, cumene peroxide and other chemicals used for enzyme assays were purchased from Sigma Chemical Company (St. Louis, MO, USA). Electrophoresis reagents were purchased from BioRad (Hercules, CA, USA), whereas antibodies for Western blot analysis were procured from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and treatment with experimental agents

A complete blood cell count was performed for each patient and control subject recruited in this study, and the total leukocyte count in blood of galactosemia patients ranged from 4.2 to 5.8×10^9/L as compared with 3.9 to 6.2×10^9/L in blood samples of healthy control subjects. Although no significant difference was observed in population of any specific leukocyte-type in blood samples of control healthy subjects and galactosemia patients, lymphocytes, which form one of the most abundant population of peripheral blood mononuclear cells, were used in this study as these leukocytes express NOX and provide a suitable model for cell culture studies due to their proliferative characteristic in comparison to monocytes that also express NOX but have a short life span and are unable to grow in cultures. Lymphocytes were isolated using the Ficoll gradient centrifugation method, and pure cultures of lymphocytes were established by culturing peripheral mononuclear cells in Dulbecco's Modified Eagles's Medium Nutrient Mixture F-12 Ham (DMEM-F12) for 5–7 days according to the method of Haller and coworkers.[18] DMEM-F12 is a galactose-deficient cell culture medium and contains 3.15 g/L of D-glucose. Established lymphocyte cultures from galactosemia patients and healthy controls were used for experiments to test the effect of galactose and Gal-1-P on NOX activity. Cell cultures were washed twice with serum-free RPMI culture medium before the start of each experiment. Cells were treated with varying concentrations (0–50 mM) of galactose and Gal-1-P (0–5.0 mM) for 3–14 days, and cultures were replenished with galactose/Gal-1-P every 24 h. In control experiments, 10–50 mM of mannitol was added to the lymphocyte cultures from control subjects for 10 days to examine its effect on NOX activity. Lipofectamine was added as a permeating agent to cell cultures in experiments with the polar molecule Gal-1-P. Gal-1-P was dissolved in culture medium supplemented with 1 mM α-cyclodextrin, and intracellular levels of Gal-1-P were periodically checked after thoroughly washing the cells to ensure permeation of the cells to Gal-1-P. Following treatment of cell cultures with experimental agents, supernatants were collected and cells were harvested to prepare homogenates for measurement of Gal-1-P, lipid peroxidation, enzymatic

activities of catalase, GPx, SOD and NOX and to perform Western blot analysis. Cells were homogenized in 50 mM of Tris-HCl buffer, pH 7.4, containing protease inhibitors.

Measurement of Gal-1-P

Levels of Gal-1-P were measured in red blood cells and homogenates of lymphocytes using a slightly modified enzymatic assay previously described by Yamaguchi and co-workers.[19]

Assay of antioxidant enzymes and lipid peroxidation

Catalase activity was assayed by measuring the degradation of hydrogen peroxide in the presence or absence of aminotriazole as described elsewhere.[20] GPx was measured in cell homogenates of lymphocytes isolated from control subjects and galactosemia patients according to the enzyme assay previously described by Reddy *et al.*[21] using cumene peroxide as a substrate. Enzymatic activity of total SOD was assayed[22] using xanthine/xanthine oxidase as source for superoxide radicals and their detection through the reduction of nitro-blue tetrazolium. One unit of SOD activity was defined as the amount of protein required for a 50% reduction in the production of superoxide anions. Malondialdehyde (MDA) levels were measured in tissue homogenates as an index of lipid peroxide production. A Calbiochem kit involving the spectrophotometric method was used to assay MDA levels.

NOX activity

NOX activity was measured in cell homogenates prepared from blood cells of galactosemia patients and control subjects using lucigenin and modification of a method described earlier by Rajagopalan *et al*.[23] Briefly, a known amount of cell homogenate was added to a reaction mixture that contained 50 mM of phosphate buffer, 0.01 mM of EDTA and 25 μM of lucigenin. Reaction was started by addition of 100 μM of NADPH, and chemiluminescence was recorded. NOX activity was calculated as relative light units emitted per milligram of protein per minute.

Assay of glucose-6-phosphate dehydrogenase enzyme activity

Glucose-6-phosphate dehydrogenase (G-6-PD) enzyme assays were performed according to the spectrophotometric method described by Beutler,[24] where G-6-PD oxidizes glucose-6-phosphate to 6-phosphogluconate, with concurrent reduction of nicotinamide adenine dinucleotide phosphate to reduced nicotinamide adenine dinucleotide phosphate, which can be measured at 340 nm. G-6-PD enzyme activity was expressed as units per milligram of protein.

Detection of nitrite

To assess the production of NO in lymphocytes of healthy subjects and galactosemia patients, nitrite levels were measured in cell homogenates using the Greiss assay as described by Furchgott and Zawadzki.[25] Cells treated with NO donor sodium nitroprusside (250 μM) were used as a positive control.

Protein content of cell homogenates was measured using Bio-Rad's protein assay kit (catalogue no. 500-0006). Protein levels of the cellular homogenates were used to normalize the data of enzyme assays by calculating the specific activity (units/milligram of protein) of various enzymes.

Western blot analysis

Polyacrylamide gel electrophoresis was performed on cell lysates prepared in Laemmeli sample buffer. Proteins were transferred onto nitrocellulose membrane following electrophoresis and subjected to Western blot analysis using polyclonal antibodies against NOX-1, p22phox, p47phox, p67phox and actin. Bands were visualized using horse radish peroxidase-labelled secondary antibody and electrochemiluminescence-based kits from Calbiochem.

Statistical analysis

Data were analysed using student's *t*-test for significance of variance between different groups of the study.

Results

Antioxidant enzymes, lipid peroxidation, nitrite production and NOX

To assess cellular oxidative stress during galactosemia, activities of antioxidant enzymes, catalase, GPX and SOD were assayed in PBL isolated from galactosemia patients and control subjects. Figure 1 shows that enzymatic activities of catalase and GPx were significantly ($P < 0.01$) reduced in galactosemia patients, whereas SOD activity remained unaltered. Levels of lipid peroxides were however significantly ($P < 0.01$) higher in cells isolated from galactosemic patients when compared with controls (Figure 1), suggesting an increased cellular oxidative stress. To examine whether the observed decrease in activities of catalase and GPX in lymphocytes isolated from

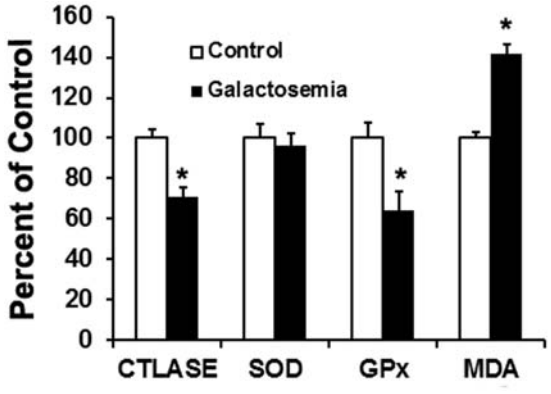

Figure 1 Specific activities of catalase (CTLASE), glutathione peroxidase (GPx), superoxide dismutase (SOD), and levels of malondialdehyde (MDA), all shown as percentage of control in lymphocytes isolated from control subjects (light bars) and galactosemia patients (dark bars). Values shown are mean ± standard deviation ($n = 15$), n is the number of samples in each group. Data were normalized with protein and show specific enzyme activities (units/mg protein). *$P < 0.01$ when compared with control subjects

galactosemia patients was in fact galactose, or Gal-1-P mediated, we measured the GPX and catalase activities in control lymphocytes cultured for 14 days in the presence of galactose and Gal-1-P. When compared with the GPX activity in untreated control cells (42.9 ± 5.7 nmoles/min/mg protein), enzyme activity was significantly ($P < 0.05$) decreased in cells following treatment with galactose (28.3 ± 7.2 nmoles/min/mg protein) as well as with Gal-1-P (24.5 ± 6.1 nmoles/min/mg protein); however, catalase activity was not markedly altered by treatment of cells with either galactose or Gal-1-P (data not shown). To investigate any role of NOX, a major enzymatic source of superoxide production, in galactosemia-induced cellular oxidative stress, we measured the enzymatic activity of NOX, and it was observed to be significantly ($P < 0.01$) reduced in lymphocytes of galactosemia patients as compared with normal control lymphocytes (Figure 2). Western blot analysis, however, revealed that the protein levels of NOX-1, the major type of NOX found in peripheral mononuclear blood cells, were not altered (as determined by the ratio of NOX-1/actin) by galactosemic conditions (Figure 2). Nitrite levels of lymphocytes isolated from galactosemia patients (1.89 ± 0.21 pmoles/mg of protein) were not significantly different from the nitrite levels in cells isolated from healthy controls (2.29 ± 0.47 pmoles/mg of protein).

Effect of galactose on NOX activity in cultured lymphocytes

In view of our observation that NOX activity is significantly reduced in lymphocytes of galactosemia patients, we cultured patients' lymphocytes and those isolated from control subjects for 3–14 days in a galactose-deficient culture medium. Figure 3 shows that NOX activity significantly increased in galactosemia lymphocytes but remained at subnormal levels when cultured in a medium with reduced galactose concentration. A statistically significant ($P < 0.05$) increase in NOX activity of galactosemic lymphocytes was observed after 10 days of culture in galactose-deficient medium, and this observed increase in NOX activity at day 10 of culture coincided with a marked reduction in Gal-1-P levels (0.012 ± 0.004 mmoles/g protein) as compared with Gal-1-P levels (0.26 ± 0.03 mmoles/g protein) at day 0 of culture. Figure 3 also shows that NOX activity in control lymphocytes was not markedly altered by decreasing galactose levels in the culture media. To further investigate the effect of galactose on NOX activity in lymphocytes, we cultured lymphocytes from patients and control subjects for two weeks in the presence of varying concentrations (0–50 mM) of galactose. Figure 4a shows that NOX activity was significantly decreased ($P < 0.01$) in lymphocytes isolated from both patients and control subjects when cultured in the presence of supra-physiological levels of galactose. Cellular levels of Gal-1-P were found to be significantly ($P < 0.01$) increased from non-detectable amounts in control lymphocytes to 0.49 ± 0.06 mmoles/g protein following treatment with 20 mM concentration of galactose (Table 1). Further, galactose-induced reduction in NOX activity was more profound (45%) in control lymphocytes as compared with the loss of NOX activity (30%) in galactosemia patients. Addition of mannitol (10 mM, 25 mM or 50 mM) to the cell cultures for 10 days did not have any significant effect on the NOX activity of lymphocytes isolated from control subjects or galactosemia patients (Figure 4b).

Gal-1-P and NOX activity

To examine a possible role of Gal-1-P in observed effects of high galactose on the enzyme activity of NOX, we measured the levels of Gal-1-P in homogenates of lymphocytes cultured in the presence of supra-physiological levels of glucose. Table 1 shows that levels of Gal-1-P were significantly higher in red blood cells of galactosemia patients as compared with those from control healthy subjects. Table 1 also shows that levels of Gal-1-P in lymphocytes increased proportionally to an increase in the amount of galactose in the culture medium. Figure 5 shows that addition of Gal-1-P (0.2–5.0 mM) to the culture medium significantly inhibited the enzymatic activity of NOX in lymphocytes from control healthy subjects.

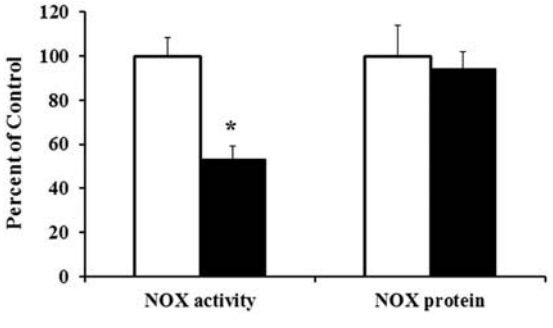

Figure 2 NOX activity and levels of NOX-1 protein (ratio of NOX-1/actin Western blots) shown as percentage of control in lymphocytes isolated from healthy controls (light bars) and galactosemia patients (dark bars). Values shown are mean ± standard deviation ($n = 15$), n is the number of samples in each group. Data were normalized with protein and show the specific enzyme activity of NOX (units/mg protein). *$P < 0.01$ when compared with control subjects

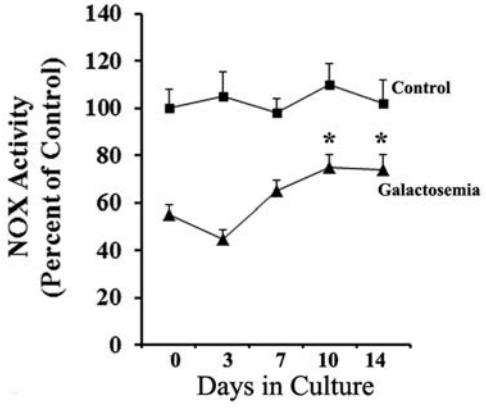

Figure 3 Enzymatic activity of NOX in lymphocytes from healthy controls (dark squares) and galactosemia patients (dark triangles) following culture in galactose-deficient medium for 3–14 days. Values shown are mean ± standard deviation of eight determinations. Data show the specific enzyme activities (units/mg protein). *$P < 0.01$ when compared with day 0 (dark triangle)

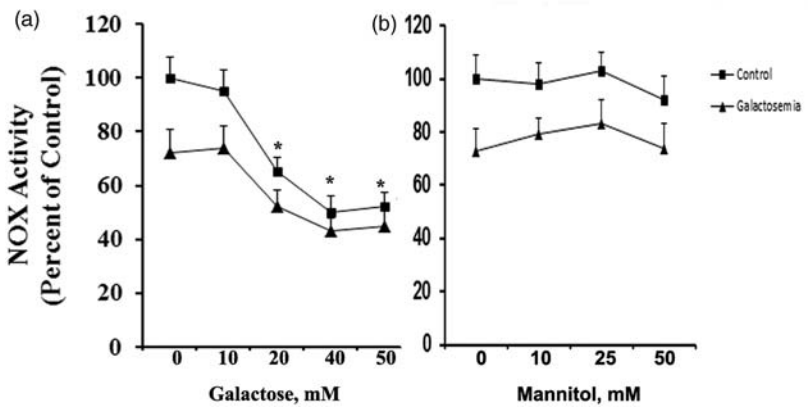

Figure 4 NOX activity in cultures of lymphocytes isolated from healthy controls (dark squares) and galactosemia patients (dark triangles) after treatment with varying concentrations (0–50 mM) of galactose (a) or mannitol (b). Values shown are mean ± standard deviation of eight determinations. Data were normalized with protein and show specific enzyme activities (units/mg protein). *$P < 0.01$ when compared with absence (0 mM) of galactose (dark triangles or squares)

Table 1 Concentration of Gal-1-P (mmoles/g cell protein) in lymphocytes from control healthy subjects and galactosemia patients

Group/cell type	Gal-1-P (mmoles/g of cellular protein)
Control (RBC)	0.011 ± 0.005
Galactosemia (RBC)	0.58 ± 0.13*
Galactosemia (lymphocytes)	0.26 ± 0.05#
Control (lymphocytes)	ND
Control lymphocytes cultured (for 14 days)	
Galactose (1 mM)	0.021 ± 0.009#
Galactose (2 mM)	0.068 ± 0.011#
Galactose (10 mM)	0.18 ± 0.07#
Galactose (50 mM)	0.49 ± 0.16#

Values are mean ± SD of six determinations.
*$P < 0.001$ when compared with control (RBC).
#$P < 0.01$ when compared with control lymphocytes.
ND, Non detectable; RBC, red blood cells.

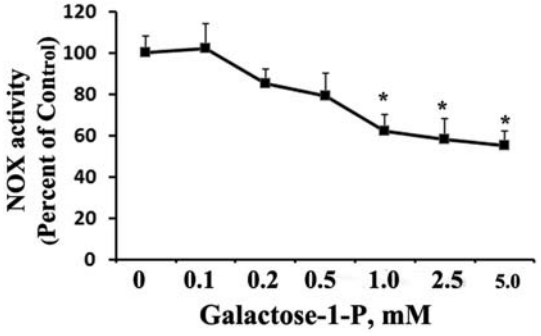

Figure 5 Effect of Gal-1-P (0.1–5 mM) on NOX activity in cultures of lymphocytes isolated from healthy controls. Values shown are mean ± standard deviation of six determinations. Data were normalized with protein and show the specific enzyme activities (units/mg protein). *$P < 0.01$ when compared with control (0 mM of Gal-1-P)

Effect of galactose and Gal-1-P on G-6-PD activity

To examine whether the effects of galactose and Gal-1-P on NOX activity are mediated through modulation of G-6-PD activity, we measured the enzyme activity of G-6-PD in control lymphocytes that were cultured in the presence of galactose (0–50 mM) or Ga-1-P (0–5 mM) for 10 days. G-6-PD activity was found to be markedly decreased in cells treated with both galactose and Gal-1-P. However, the statistically significant ($P < 0.05$) inhibition of G-6-PD activity was only observed in cells that were treated with the highest concentrations, 50 mM of galactose and 5 mM, of Gal-1-P when compared with enzyme activity in untreated control cells (Figure 6).

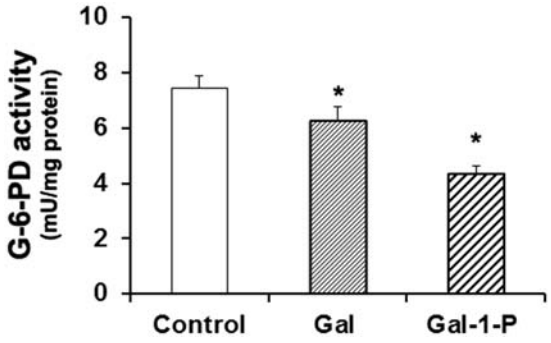

Figure 6 Effect of galactose (50 mM) and Gal-1-P (5 mM) on G-6-PD activity in cultures of control lymphocytes. Data show specific enzyme activities (milliunits/mg protein) and values are mean ± standard deviation of six determinations. *$P < 0.05$ when compared with control (absence of galactose and Gal-1-P)

Effect of galactose and Gal-1-P on NOX-1, p22phox, p47phox and p67phox in cultured lymphocytes

To further investigate the molecular basis of Galactose- or Gal-1-P-mediated inhibition of NOX activity in cultured lymphocytes, Western blotting was performed to assess the protein levels of NOX-1, p22phox, p47phox and p67phox in cultured lymphocytes isolated from control healthy subjects. Figure 7 shows that the levels of NOX-1, p22phox and p67phox in cultured lymphocytes were not affected by high galactose or Gal-1-P. Levels of p47phox were markedly decreased in lymphocytes cultured in the

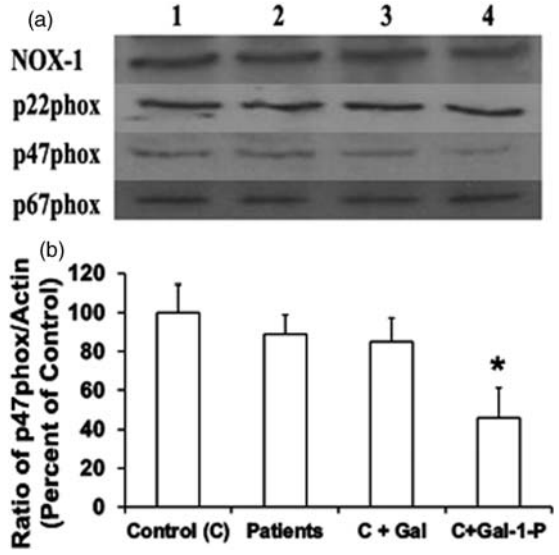

Figure 7 Western blot (a) showing NOX-1, p22phox, p47phox, p67phox and actin proteins in cell lysates prepared from lymphocytes isolated from control healthy subjects (lane 1), galactosemia patients (lane 2), and control lymphocytes cultured for 2 weeks in the presence of 20 mM galactose (lane 3) or 1.0 mM Gal-1-P (lane 4). The bar graph (b) shows the ratio of p47 phox and actin (as percentage of control). *$P < 0.01$ when compared with the control group (lane 1)

presence of Gal-1-P, whereas p47phox remained unaltered in lymphocytes cultured in a high galactose-containing medium. Interestingly, levels of NOX-1, p22phox, p47phox and p67phox in lymphocytes from galactosemic patients were not significantly different from the ones in lymphocytes from healthy control subjects.

Discussion

This study shows for the first time that NOX, a major source of superoxide production in eukaryotic cells, is impaired in galactosemia patients and provides new insights into the molecular mechanisms of high galactose-induced cellular pathology and development of the disease.

Galactosemia is an autosomal recessive disorder with well-characterized clinical features. Although an increased risk of *E. coli* infections[2,26] in galactosemia patients has been widely reported, the underlying mechanisms have remained unclear. Investigations into the pathogenic mechanisms of galactosemia point towards galactose-induced complications, although the effects of restoration of normal galactose plasma levels by dietary lactose restrictions on the course of disease progression remain debatable.[27,28] Others have reported that besides increased plasma concentrations of galactose, high levels of Gal-1-P in the blood of galactosemia patients trigger cellular damage through various modifications at the molecular level. All patients in this study were non-compliant to the lactose-free diet as supported by a marked elevation in the serum levels of liver enzymes, and it appears that non-compliance to diet modifications might be responsible, at least in part, for the observed markedly elevated levels of Gal-1-P in our patients. Several molecular and cellular studies have indicated an important role of Gal-1-P in the development of cellular oxidative stress during pathogenesis of galactosemia.[7,29] Increased lipid peroxidation and decreased levels of antioxidant enzymes have been reported to occur in galactosemic tissues,[30] and our results that galactose and Gal-1-P markedly inhibit GPX activity support the earlier findings. Galactose- or Gal-1-P-mediated decrease in the enzymatic activity of SOD in retina and other tissues has been reported earlier, and our finding that lymphocytes from galactosemia patients have unaltered SOD levels could be tissue specific.[31] Our observations in this study that catalase and GPx activities are reduced in lymphocytes along with an increase in lipid peroxide levels in galactosemia lymphocytes support earlier findings. However, normal enzymatic activity of SOD in lymphocytes of galactosemia patients in this study suggests that superoxide-mediated mechanisms might not be participating in high galactose-induced development of cellular oxidative stress. This notion is further supported by our findings that enzymatic activity of NOX, a major source of cellular superoxide production, is in fact impaired in galactosemic lymphocytes. These findings clearly suggest that cellular oxidative stress in galactosemia is mainly due to decreased antioxidant enzyme activities and is not NOX mediated. Although hyperglycaemia and diabetes have been shown to induce NOX activity[32,33] an effect of galactose on NOX activity has not been reported as yet. Our cell culture studies show for the first time that high levels of galactose and Gal-1-P significantly inhibit NOX activity in lymphocytes isolated from control healthy subjects and galactosemia patients. Our findings that NOX activity is partially restored in lymphocytes from galactosemia patients when cultured in galactose-deficient conditions indicate that the observed impairment of NOX activity is galactose induced. The fact that supra-physiological concentrations of galactose or Gal-1-P, levels not observed in *in vivo* galactosemic conditions, are required to inhibit NOX activity significantly in cultured lymphocytes could possibly be attributed partly to an active catabolism of galactose in healthy normal lymphocytes or *in vitro* conditions that might impair uptake of galactose by the cells in culture. However, in comparison with galactose, a markedly lower concentration of Gal-1-P was observed to inhibit NOX activity in cultured lymphocytes, suggesting that intracellular concentration of Gal-1-P is a determining factor for inhibition of NOX activity irrespective of the extracellular levels of galactose. A metabolic enigma with respect to different intracellular or extracellular concentrations of galactose and Gal-1-P has been described earlier in galactosemic patients.[34] The fact that levels of NOX-1 protein remain unaltered in lymphocytes of galactosemia patients, as observed in this study, would infer that galactose- or Gal-1-P-mediated impairment of NOX is likely through a post-translational modulation or an impaired function of its other cytosolic components – p22phox, p47phox and/or p67phox. Our observation that the levels of p47phox were markedly reduced by high levels of Gal-1-P in lymphocyte cultures from healthy individuals suggests that the observed impairment of NOX activity in galactosemia patients may partly be due to interference of Gal-1-P in interaction between p47phox and catalytic component

NOX-1. Further, high levels of Gal-1-P in the blood of galactosemia patients have been shown earlier to inhibit pentose cycle enzymes, G-6-PD and 6-phosphogluconate dehydrogenase, which are responsible for cellular production of NADPH.[23] An abnormally high intracellular concentration of Gal-1-P in lymphocytes from galactosemia patients and inhibition of lymphocytic G-6-PD activity by galactose and Gal-1-P as observed in this study strongly suggests that a reduced production of NADPH under galactosemia conditions, and hence its deficiency, could be another possible cause for impairment of cellular NOX activity.

Although overproduction of superoxide anions through the induction of the NOX system has been widely reported in pathological conditions such as atherosclerosis and diabetes, NOX is also known to regulate several physiological functions through gradual and periodic production of superoxide anions.[35,36] Peroxinitrite generation through an interaction of superoxide anions with NO further underscores the role of NOX activity in pathogenic conditions. Because NO levels of galactosemic lymphocytes were found to be similar to those of control lymphocytes in our study, NOX-mediated effects in galactosemia lymphocytes would be expected to get predominantly executed through superoxide anions. Phagocytes are well known to act against invading pathogens through production of superoxide anions by phagocytic NOX.[37,38] Decreased NOX activity in peripheral blood cells would indicate an inefficient host defence system against invading organisms. Kobayashi and coworkers reported earlier that galactose inhibits neutrophil function in neonates, and the bactericidal activity of neutrophils was markedly decreased following incubation with supra-physiological amount of galactose.[16] In view of our current knowledge that phagocytes are equipped with NOX system to generate superoxide anions and kill bacteria, we can suggest that an impaired NOX system under galactosemic conditions as observed in this study could possibly be responsible, at least in part, for the reported inhibitory action of galactose on the capability of phagocytes to kill *E. coli in vitro*.[17] Besides acting as a regulator of cellular redox, NOX is also known to mediate several other physiological activities such as tonicity of vascular smooth muscle cells. Decreased NOX activity in galactosemia patients could possibly be responsible for other clinical complications as well. In conclusion, the observed impairment of NOX in PBL of galactosemia patients is likely Gal-1-P mediated and provides a new insight into the cellular and molecular mechanisms of its pathogenesis.

Author Contributions: This study was conducted with significant contribution from all authors. MA and GSD planned the study; WA and MA recruited patients in this study. GD and IK carried out the biochemical and cell culture experiments. GD and MA analysed the data, and all authors contributed in preparing the manuscript.

ACKNOWLEDGEMENTS

The authors are thankful to Mrs. Wisam Muawad, Mrs. Taqdees Aqeel and Mr. B. Srivastava for their technical support. This study was carried with financial support from Kuwait University, Kuwait.

REFERENCES

1. Bosch AM, Grootenhuis MA, Bakker HD, Heijmans HS, Wijburg FA, Last BF. Living with classical galactosemia, health related quality of life consequences. *Pediatrics* 2004;**113**:e423–8
2. Segal S. Komrower Lecture. Galactosemia today: the enigma and the challenge. *J Inherit Metab Dis* 1998;**21**:455–71
3. Landt M, Ritter D, Lai K, Benke PJ, Elsas LJ, Steiner RD. Black children deficient in galactose 1-phosphate uridyltransferase: correlation of activity and immunoreactive protein in erythrocytes and leukocytes. *J Pediatr* 1997;**130**:972–80
4. Wehrli SL, Reynolds R, Chen J, Yager C, Segal S. Metabolism of 13C galactose by lymphoblasts from patients with galactosemia determined by NMR spectroscopy. *Mol Genet Metab* 2002;**77**:296–303
5. Lai K, Elsas LJ. Structure-function analysis of a common mutation in blacks with transferase-deficiency galactosemia. *Mol Genet Metab* 2001;**74**: 264–72
6. Leslie ND. Insights into the pathogenesis of galactosemia. *Annu Rev Nutr* 2003;**23**:59–80
7. Schulpis KH, Papassotiriou I, Tsakiris S. 8-hydroxy-2-desoxyguanosine serum concentrations as a marker of DNA damage in patients with classical galactosemia. *Acta Paediatrica* 2006;**95**:164–9
8. Banerjee AK, Mandal A, Chanda D, Chakraborti S. Oxidant, antioxidant and physical exercise. *Mol Cell Biochem* 2003;**253**:307–12
9. Yang H, Zhou L, Wang Z, Roberts LJ 2nd, Lin X, Zhao Y, Guo Z. Over expression of antioxidant enzymes in Apo E-deficient mice suppresses benzo(a)pyrene-accelerated atherosclerosis. *Atherosclerosis* 2009;**207**:51–8
10. Vilaseca-Buscà MA, Artuch-Iriberri R, Colome-Mallolas C, Brandi-Tarrau N, Campistol J, Pineda-Marfá M, Sierra-March C. Abnormal antioxidant system in inborn errors of intermediary metabolism. *Rev Neurol* 2002;**34**:1021–4
11. Ellis NA, Sengupta N, Caballero S, Guthrie SM, Mames RN, Grant MB. Nitric oxide synthases modulate progenitor and resident endothelial cell behavior in galactosemia. *Antioxid Redox Signal* 2005;**7**:1413–22
12. DeLeo FR, Quinn MT. Assembly of the phagocyte NADPH oxidase: molecular interaction of oxidase proteins. *J Leukoc Biol* 1996;**60**:677–89
13. Quinn MT, Ammons MC, Deleo FR. The expanding role of NADPH oxidases in health and disease: no longer just agents for death and destruction. *Clin Sci* 2006;**111**:1–20
14. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 2000;**86**:494–501
15. Sorescu D, Szocs K, Griendling KK. NADP(H) oxidases and their relevance to atherosclerosis. *Trends Cardiovasc Med* 2001;**11**:124–31
16. Kobayashi RH, Kettelhut BV, Kobayashi AL. Galactose inhibition of neonatal neutrophil function. *Pediatr Infect Dis* 1983;**2**:442–5
17. Barr PH. Association of *Escherichia coli* sepsis and galactosemia in neonates. *J Am Board Fam Pract* 1992;**5**:89–91
18. Haller D, Blum S, Bode C, Hammes WP, Schiffren EJ. Activation of human peripheral blood mononuclear cells by non-pathogenic bacteria in vitro: evidence of NK cells as primary targets. *Infect Immun* 2000;**68**:752–9
19. Yamaguchi A, Fukushi M, Mizushima Y, Shimizu Y, Takasugi N, Arashima S, Ohyanagi K. Microassay for screening newborns for galactosemia with use of a fluorometric microplate reader. *Clin Chem* 1989;**35**:1962–4
20. Baudhuin P, Beaufay Y, Rahman Y, Sellinger OZ, Wattiaux R, Jacques P, De Duve C. Tissue fractionation studies. *Biochem J* 1964;**92**:179–84
21. Reddy CC, Tu C, Burgess JR, Ho CY, Scholz RW, Massaro EJ. Evidence for the occurrence of selenium-independent glutathione peroxidase activity in rat liver microsomes. *Biochem Biophys Res Commun* 1981;**101**:970–8
22. Crapo JD, McCord JM, Fridovich I. Preparation and assay of superoxide dismutases. *Methods Enzymol* 1978;**53**:382–93

23. Rajagopalan S, Kurz S, Münzel T, Tarpey M, Freeman BA, Griendling K. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 1996;**97**:1916–23
24. Beutler E. *Red Cell Metabolism: A Manual of Biochemical Methods*. 3rd edn. Grune and Stratton, New York, NY: 1984:68–71
25. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature* 1980;**288**:373–6
26. Shurin SB. *Escherichia coli* septicemia in neonates with galactosemia. *N Engl J Med* 1977;**297**:1403–4
27. Chung MA. Galactosemia in infancy: diagnosis, management and prognosis. *Pediatr Nurs* 1997;**23**:563–9
28. Widhalm K, Miranda da Cruz BD, Koch M. Diet does not ensure normal development in galactosemia. *J Am Coll Nutr* 1997;**16**:190–1
29. Gityzelman R. Galactose-1-phosphate in the pathophysiology of galactosemia. *Eur J Paediatr* 1995;**154**:S45–9
30. Otsyula M, King MS, Ketcham TG, Sanders RA, Watkins JB III. Oxidative stress in rats after 60 days of hypergalactosemia or hyperglycemia. *Int J Toxicol* 2003;**22**:423–7
31. Kowluru RA, Kern TS, Engerman R. Abnormalities of retinal metabolism in diabetes or experimental galactosemia. IV. Antioxidant defense system. *Free Radic Biol Med* 1997;**22**:587–92
32. Block K, Gorin Y, Abboud HE. Subcellular localization of Nox4 and regulation in diabetes. *Proc Natl Acad Sci USA* 2009;**106**:14385–90
33. Matsushima S, Kinugawa S, Yokota T, Inoue N, Ohta Y, Hamaguchi S, Tsutsui H. Increased myocardial NAD(P)H oxidase-derived superoxide causes the exacerbation of postinfarct heart failure in type 2 diabetes. *Am J Physiol Heart Circ Physiol* 2009;**297**:H409–16
34. Ning C, Segal S. Plasma galactose and galactitol concentration in patient with galactose-1-phosphate uridyltransferase deficiency galactosemia: determination by gas chromatography/mass spectrometry. *Metabolism* 2000;**49**:1460–6
35. Delbosc S, Cristol JP, Descomps B, Chenard J, Sirois P. Oxygen and superoxide anion Modulation of NADPH oxidase? *J Soc Biol* 2001;**195**:401–11
36. Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002;**82**:47–95
37. Arruda MA, Barja-Fidalgo C. NADPH oxidase activity: in the crossroad of neutrophil life and death. *Front Biosci* 2009;**14**:4546–56
38. Dale DC, Boxer L, Liles WC. The phagocytes: neutrophils and monocytes. *Blood* 2008;**112**:935–45

(Received June 26, 2012, Accepted January 17, 2013)