JBACHS
Journal of Basic and Clinical Health Sciences

J Basic Clin Health Sci 2020; 4:230-236
https://doi.org/10.30621/jbachs.2020.1057

Original investigation

Assessment of Rac1 and β-PAK Expressions in a Mouse Model for Contrast-Induced Nephropathy

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Received: 06.12.2020; **Accepted:** 30.04.2020; **Available Online Date:** 15.10.2020

Cite this article as: Manisaligil YA, Cilaker Micili S, Kizildag S, Celik A, Bagriyanik HA, Gumustekin M. Assessment of Rac1 and β-PAK Expressions in a Mouse Model for Contrast-Induced Nephropathy. J Basic Clin Health Sci 2020; 4:230-236.

ABSTRACT

Purpose: Radiocontrast-induced nephropathy is an important clinical problem in high-risk patients. The mechanism of cytotoxic effect of radiocontrast media on kidneys is still not fully clarified. Rac1/β-PAK pathway has been shown to play a role in other nephropathies such as diabetic nephropathy. In this study, it was aimed to determine whether Rac1/β-PAK signalling pathway has any role in the development of contrast-induced nephropathy (CIN).

Methods: Adult male Balb/C mice were used. A single dose of iohexol was given intraperitoneally. Thirty-two mice were divided into 5 groups including control (Group1, n=4), pretreatment (Group 2, n=7), low dose (2 g iodine/kg) iohexol (Group 3, n=7), medium dose (2.5 g iodine/kg) iohexol (Group 4, n=7) and high dose (3 g iodine/kg) iohexol (Group 5, n=7). The animals were sacrificed 24 hours after iohexol administration. Nephropathy were evaluated by histological and biochemical analysis methods. Expressions of Rac1 and β-PAK were evaluated by immunohistochemical method. Apoptosis was assessed by TUNEL method.

Results: It was found that severity of nephropathy, apoptosis and β-PAK expressions significantly increased as iohexol dosage increased. Although Rac1 expression was higher in the iohexol group in comparison to the control and pretreatment groups, its increase did not show a dose-dependent manner. There was no significant difference among the groups in terms of serum creatinine. Serum cystatin C were increased in all groups compared to the control group, but significant increase was observed only in the pretreatment group.

Conclusions: Our findings suggest that the Rac1/β-PAK signal transduction pathway may have a role in the contrast-induced nephropathy model.

Keywords: apoptosis, contrast-induced nephropathy, iohexol, mouse, Rac1

INTRODUCTION

Today, the advancement of technology in radiological imaging systems and the development of new imaging methods increased the use of radiocontrast media (CM) (1). In patients who were administered intravascular CM injections, nephropathy was observed to develop in about 15% of those with no chronic disease and in 50% with chronic kidney disease, diabetes, heart failure and anemia (2). Intracellular energy loss, deterioration of calcium balance, change in polarity of tubular cells, formation of reactive oxygen species (ROS), activation of caspases and apoptosis are some of the renal cytotoxic effects caused by CM injections (3). In studies performed in risk-bearing patient groups regarding nephropathy, CM administration was shown to increase ROS formation in tubular cells by decreasing renal blood flow (4). Although the mechanism of nephrotoxicity due to CM administrations has not been totally clarified yet; various factors such as increased osmotic load, vasoconstriction in renal arteries and decreased renal blood flow have been suggested so far.

Ras-related C3 botulinum toxin substrate 1 (Rac1) is a small protein molecule belonging to the Rho subgroup of the GTPase family, weighing 21 kD. Rac1 is also a subunit of NADPH oxidase enzyme and has a role in intracellular formation of ROS. For the activation, Rac1 needs to be bound by GTP (5). The most important effector molecule of Rac1GTP is p21-activated kinases (PAKs). Rac1-GTP activates PAKs and eventually contributes or directly induces many intracellular events such as development of regulation

of cytoskeleton, cell-cell adhesion, actin-related motility, gene transcription, and apoptosis (6, 7). Rac1/β-PAK pathway has been shown to play a role in some clinical conditions as well as some nephropathies (8–10). We have already demonstrated in our previous study that Rac1 had a role in the development of diabetic nephropathy (8). However, the mechanism of cytotoxic effect of CM on kidneys is still not fully clarified. Therefore, this study was designed to fill the gap in this field in the literature and to demonstrate the possible link between Rac1/β-PAK signaling pathway and contrast-induced nephropathy (CIN).

MATERIALS and METHODS

Animals

Adult, male Balb/C mice (n=32) weighing 25–30 g were used in this study. The Ethics Committee of the Research of Laboratory Animals, Dokuz Eylül University Medical School (69/2012), approved the study and the 'National Institute of Health' performed all procedures according to the 'Principles of Laboratory Animal Care' published. All animals were kept in standardized conditions of temperature (22±2°C) and illumination (12:12 light/dark) and cages with mesh bottoms providing free access to tap water and pelleted food until 16 hours prior to the commencement of the experiment.

Experimental groups

In the literature, the dose of iodine used to produce CIN is between 1.5–10 g iodine/kg (7, 11). The most commonly used doses are 2–3.5 g iodine/kg (12, 13). Therefore, we preferred to use this dose range in our study. CIN model in mice is described below and shown in Figure 1.

Mice were randomly divided into 5 groups (n=32).

Group 1 (control): Nothing done to assess the basal status of mice (n=4)

Group 2 (pretreatment): indomethacin + L-NAME + equal volume saline (i. p.) as iohexol (n=7)

Group 3 (low dose iohexol): indomethacin + L-NAME + 2 g iodine/kg iohexol, i. p (n=7)

Group 4 (medium dose iohexol): indomethacin + L-NAME + 2.5 g iodine/kg iohexol, i. p (n=7)

Group 5 (high dose iohexol): indomethacin + L-NAME + 3 g iodine/kg iohexol, i. p (n=7)

CIN was induced in mice as previously described by Billings et al (7). After 16-hour dehydration period, all groups except Group 1 (control), were pre-treated with indomethacin (10 mg/kg dissolved in dimethyl sulfoxide (DMSO), i. p) and NG-nitro-Larginine methyl ester (L-NAME, 10 mg/kg dissolved in 0.9% saline, i. p) for inhibition of cyclooxygenase and nitric oxide synthase, respectively. Because iohexol is low-osmolar monomeric iodinated radiocontrast medium, the incidence of CIN complication after iohexol is low. All these procedures (reducing renal blood flow by dehydration and vasoconstriction by inhibition cyclooxygenase and nitric oxide synthase) facilitates iohexol-induced nephropathy. Therefore, 15 min after pretreatment, animals (except Group 1 and Group 2) were treated with iohexol (Omnipaque, 2; 2.5 or 3 g iodine/kg). Group 2 (pretreatment group) received equal volume saline (i. p), instead of iohexol. After injections, mice were given ad libitum access to food and water. They were sacrificed with CO_2 in 24 hours and blood samples were obtained.

This model reliably produces nephropathy following radiocontrast injection and has been previously validated in mice and rats (7, 14). Then left kidneys, upper part of abdominal aorta and left testicular tissues of mice were taken into 10% formaldehyde for histological and immunohistochemical studies. Aortic and testicular paraffin blocks were stored for further studies.

Drugs

Iohekzol (Omnipaque 300, 00666108–95–0, Turkey) was purchased from Opakim. Indomethacin (53–86–1, USA), L-NAME (51298–62–5 Shanghai, China) and DMSO (67–685, USA) were purchased from Sigma-Aldrich. Rac-1 antibody (sc-217, Santa Cruz, USA) and β-PAK antibody (sc-1871, Santa Cruz, USA) were purchased from Biotechnology.

Histomorphological examination

The kidney tissues were fixed by 10% buffered formalin for 48 h and they were embedded in paraffin. Paraffin blocks were placed in a rotary microtome (RM 2255, Leica, Germany) and sections of 5 mm thickness were obtained (13). After deparaffinization and rehydration, all sections were stained with hematoxylin and eosine (H&E) for histological examination of kidney tissues.

In order to determine nephropathy, we investigated histological changes including inflammation, tubular dilatation, glomerular injury, proteinaceous material aggregation, vacuolization (15). The abnormal tubular histology was scored and graded by a semi-quantitative score from 0 to 4 points: 0, no abnormalities; 1 +, changes affecting less than 25% of the sample; 2 +, changes affecting 25–50% ; 3 +, changes affecting 50–75% ; 4 +, changes affecting more than 75% (13).

Immunohistochemical staining

Antibody Rac-1 (sc-217, Santa Cruz Biotechnology, 1/100 dilution) and β-PAK (sc-1871, Santa Cruz, Biotechnology, 1/100 dilution) were performed. Immunostaining was performed as described previously. Immunostaining intensity was determined with H-score. The H-score value was derived for each specimen by calculating the sum of the percentage of cells. Categorized by intensity of staining, multiplied by its respective score, by means of the formula:

$$\text{H-score}=\sum \text{Pi}\ (i+1)$$

Where i ¼ intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong, respectively) and Pi is the percentage of stained cells for each intensity, varying from 0% to 100%. For each slide, five different fields were evaluated microscopically at 200x magnification. H-score evaluation was performed by at least two investigators independently (SCM and AB), blinded to the source of the samples as well as to each other's results and the average score was utilized (16).

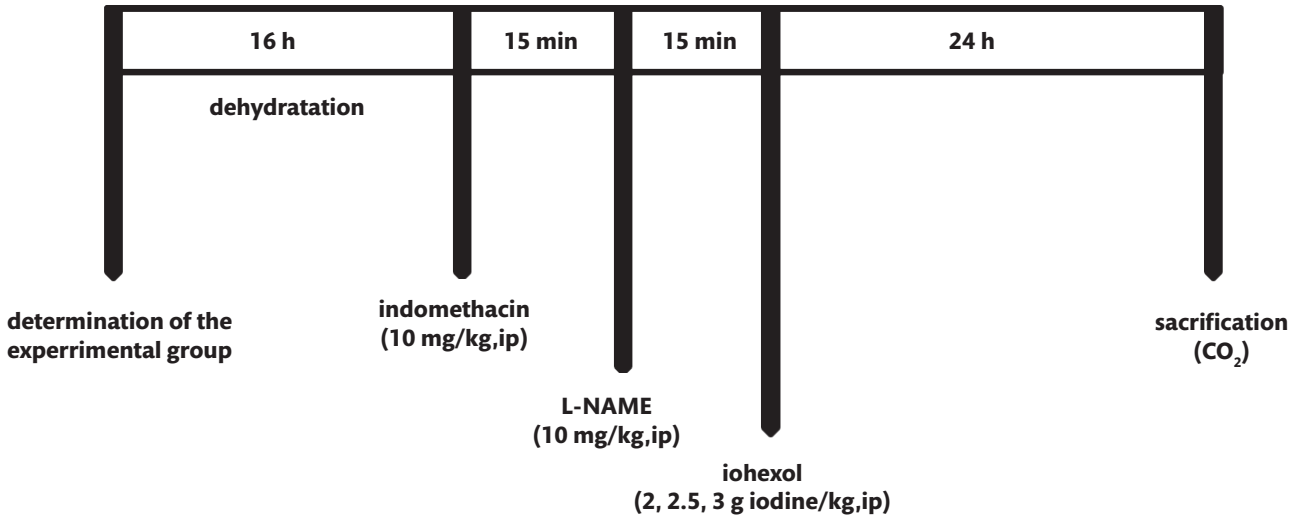

Figure 1. Contrast-induced nephropathy model in mice

Table 1. Rac1 and β-PAK immunoreactivity H-scores of experimental groups

Groups	Rac1	β-PAK	N
Control (Group1)	125±0.81	101±0.81	4
Pretreatment (Group2)	181.2±2.2	220.4±0.54	7
Low dose iohexol (Group3, 2 g iodine/kg)	272±1.8*	221±1.1	7
Medium dose iohexol (Group4, 2.5 g iodine/kg)	256±1.2*	245.2±0.75*	7
High dose iohexol (Group5, 3 g iodine/kg)	224.7±0.8*	256.2±1.3*	7

*Increased *vs* control group (p<0.05). Results are mean (± S. E. M.).

In situ cell death detection (TUNEL staining)

Apoptosis was evaluated by the in situ terminal-deoxynucleotidyl-transferase-mediated dUTP digoxigenin nick end labeling (TUNEL) assay. A Dead End Colorimetric TUNEL system kit (In Situ Cell Death Detection Kit1Roche, Mannheim, Germany) was used for apoptotic cell detection (17). The percentage of TUNEL (+) cells was determined by counting the positive cells from 5 random fields in each kidney. Two independent blinded observers performed all measurements for the source of kidney tissues.

Biochemical analysis

Serum creatinine analysis was made using a colorimetric method of Jaffe reaction (Cayman's, catalog no: 700460, USA) and Cystatin C analysis was made using Sandwich ELISA method (R&D Systems Mouse Cystatin C, Catalog no: DY1238, USA). Both analyses were performed according to manufacturer's procedures.

Statistical analysis

All values were expressed as the mean ± standard error (mean ± SEM). Statistical comparisons among the biochemical parameters of groups were analysed by two-way ANOVA and the post hoc Bonferroni test. The Kruskal-Wallis and Mann-Whitney U test were used to compare Rac1 and β-PAK immunostaining intensity values between groups. All statistical procedures were performed by SPSS 15.0 software for Windows (Chicago, IL, USA). A value of p<0.05 was considered significant.

RESULTS

Histological evaluation of contrast induced nephropathy

Inflammation was found to be increased in all groups in comparison to the control group (Group 1: 1.0±0.6), but the increase in medium- and high-dose iohexol groups (Group 4: 1.9±0.4 and Group 5: 2.4±0.2) was statistically significant (p<0.05, Figure 2, Figure 3a). Tubular dilatation determined in the high-dose iohexol group (Group 5: 2.6±0.5) was significantly increased with respect to the control group (Group 1: 1.0±0.5) (p=0.00, Figure 2, Figure 3a). In addition to tubular damage, glomerular damage was detected in medium– and high-dose iohexol groups (Group 4: 0.3±0.2 and Group 5: 0.6±0.2). Accumulation of proteinaceous substances was observed in all groups except for control (Group 1) and low-dose iohexol (Group 3) groups ((Group 2: 0.5±0.1, Group 4: 0.1±0.1 and Group 5: 0.4±0.2), but there was no statistically significant difference among groups in terms of proteinaceous substances (Figure 2, Figure 3a). Tubular edema, vacuolization and loss of brush border showed an increase in line with increasing iohexol doses, but a significant increase was detected only in high-dose iohexol group (Group 5: 1.5±0.1) in comparison to all the other groups (p<0.05, Figure 2, Figure 3a). These histomorphological changes are similar to the changes in human radiocontrast nephropathy (8).

Expressions of Rac1/β-PAK following radiocontrast exposure

Rac1 expression increased in all groups compared to the control group (Table 1). The increase was statistically significant in all

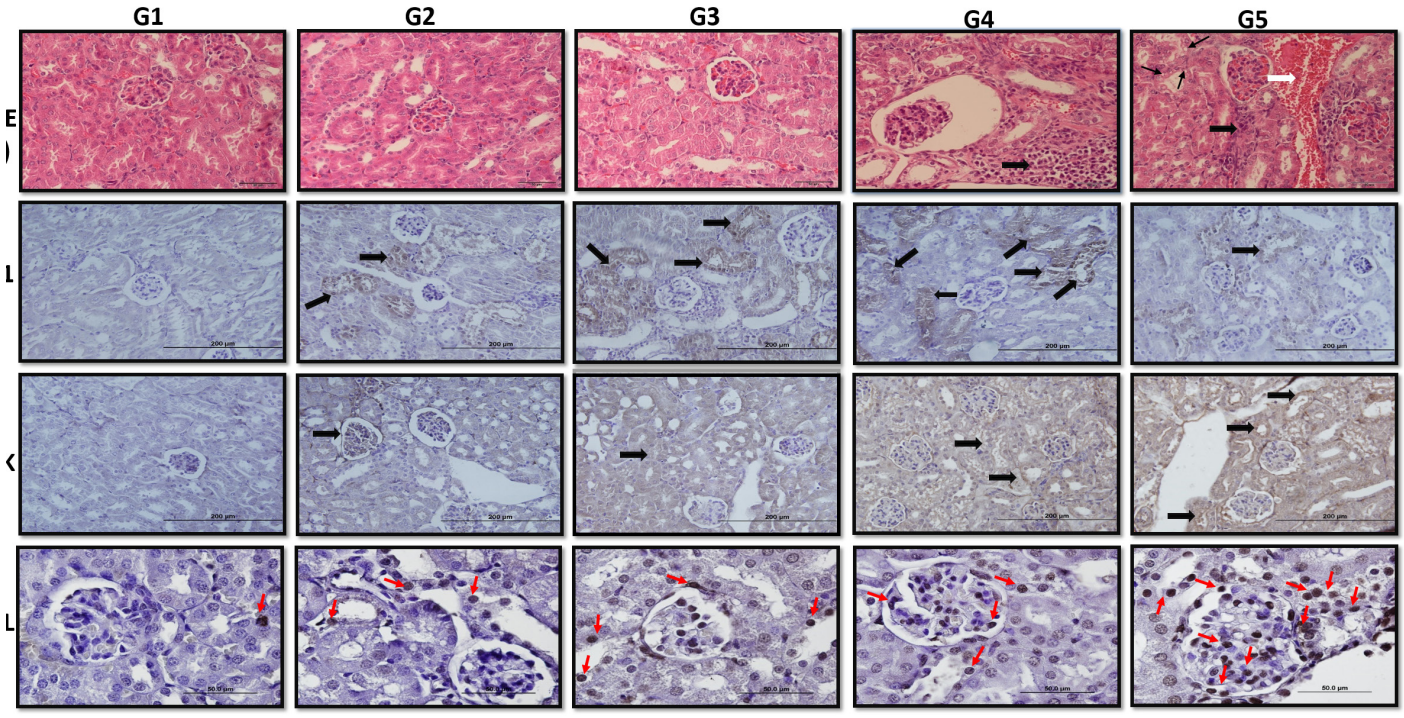

Figure 2. Representative histochemical H&E staining micrographs, Immunohistochemical Rac1, β-PAK expressions and TUNEL images of experimental group. Group 1 (control), Group 2 (pretreatment), Group 3 (low dose iohexol), Group4 (medium dose iohexol) and Group5 (high dose iohexol) groups. 1. The black arrows show inflammation, white arrow shows tubular dilatation and thin black arrows show vacuolization in tubules. 2–3. The black arrows show immunopositive cells. 4. The red arrows Show TUNEL positive cells

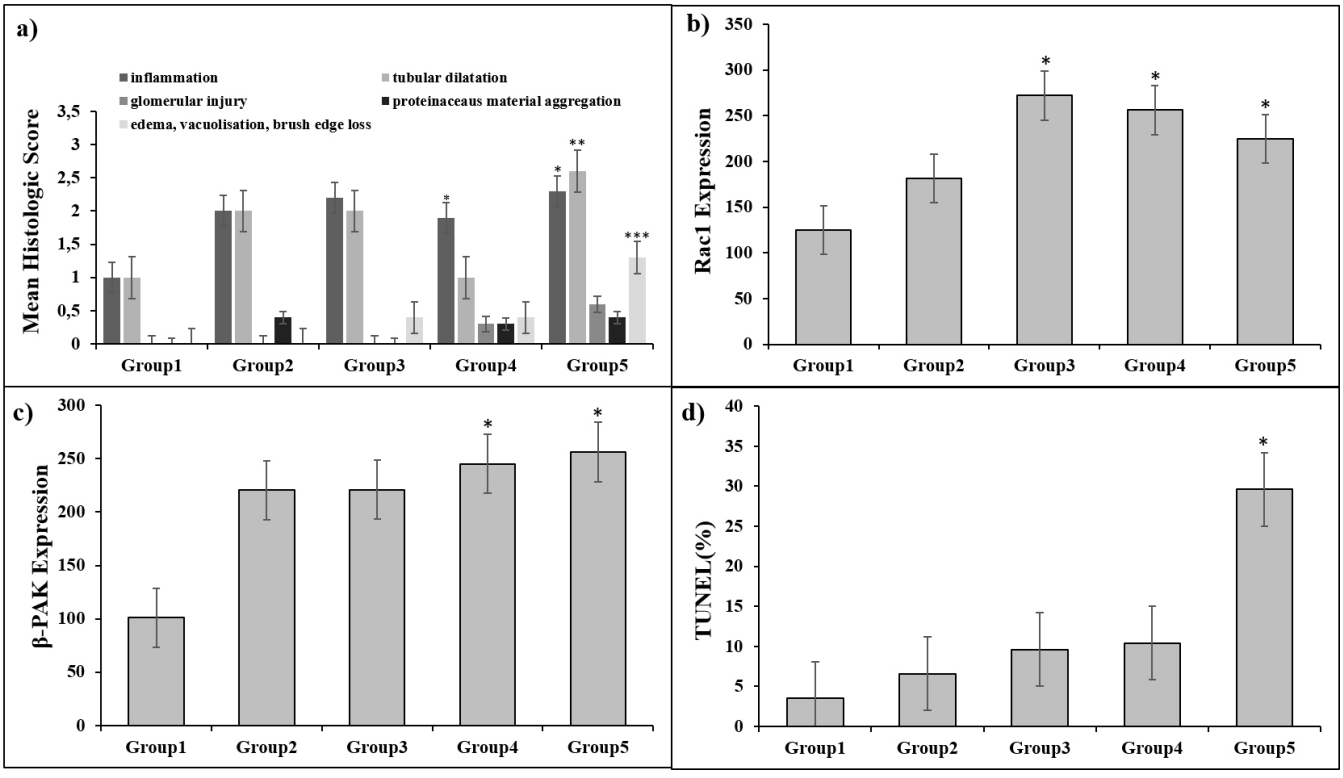

Figure 3. a) Graph bar of light microscopic nephropathy results. Results are mean (± S. E. M.). *: Inflammation were increased in Group4 (medium dose iohexol) *vs* Group1 (control) (p<0.05); *, **, ***: inflammation, tubular dilatation and vacuolization were increased Group5 (high dose iohexol) *vs* Group 1 (control) (p<0.05). **b)** Graph bar indicating the iohexol induced Rac1 expressions were inversely related to iohexol concentrations in the kidney tissues. *: (p<0.05). Results are mean (± S. E. M.). **c)** Graph bar of TUNEL positive cells ratio (%) in kidney sections from experimental groups. Results are mean (± S. E. M.). *: Apoptosis was increased in Group5 (high dose iohexol) *vs* Group 1 (control), Group 2 (pretreatment), Group3 (low dose iohexol) and Group4 (medium dose iohexol) (p<0.05). **d)** Graph bar indicating iohexol-induced β-PAK expressions were increased in Group3 (low dose iohexol), Group4 (medium dose iohexol) and Group 5 (high dose iohexol) compared than Group 1 (control). *: (p<0.05) significantly increased, results are mean (± S. E. M.).

Table 2. Serum creatinine and cystatin C levels of experimental groups

Groups	Creatinine (mg/dL)	Cystatin C (pg/mL)	N
Control (Group1)	1.65±0.27	759.80±36.83	4
Pretreatment (Group2)	1.63±0.23	1255.90±71.22*	4
Low dose iohexol (Group3, 2 g iodine/kg)	1.94±0.52	1174.96±155.09	4
Medium dose iohexol (Group4, 2.5 g iodine/kg)	1.25±0.49	938.71±61.91	5
High dose iohexol (Group5, 3 g iodine/kg)	1.39±0.21	1077.56±106.62	6

*Increased *vs* control group (p<0.05). Results are mean (± S. E. M.).

iohexol administered groups (Groups 3, 4 and 5) (p<0.05, Figure 2 and Figure 3b). β-PAK expressions in iohexol groups were observed to increase dose-dependent manner. β-PAK expression increased in all groups with respect to the control (Group1) group. However, the increase was found to be statistically significant in medium and high dose iohexol groups (Group 4 and 5) (p<0.05, Figure 2 and Figure 3 c).

Determination of Apoptotic Cells in CIN

The ratio of TUNEL positive cells was increased in all groups in comparison to the control group (Group1), but only the high-dose iohexol group (Group5) showed statistically significant increase (p<0.05, Figure 2 and Figure 3 d).

Biochemical analysis

The highest serum creatinine level (1.94±0.52 mg/dL) was observed in the low dose iohexol group (Group 3). However, there was no significant difference between the groups in terms of serum creatinine levels (Table 2). Serum cystatin C levels were increased in all groups in comparison to the control (Group 1) group. The level of cystatin C was significantly increased in the pretreatment (Group 2) group compared to the control (Group 1) group (1255.90 pg/mL *vs* 759.80±36.83 pg/mL, p<0.05, Table 2).

DISCUSSION

In our study investigating the role of Rac1/β-PAK signaling pathway in the pathogenesis of CIN, we demonstrated that, increasing dose of iohexol had an increasing effect on the severity of nephropathy, apoptosis and β-PAK expressions, dose-dependent manner. Although increased at a certain level, Rac1 expressions however, did not show a dose-dependent pattern in iohexol groups. Pretreatment with indomethacin (10 mg/kg, ip) and L-NAME (10 mg/kg, ip) was proved to increase the severity of nephropathy by reducing renal medullary blood flow in previous studies (3, 10, 13) Consistent with previous studies, in our study, the signs of inflammation, tubular dilatation, and accumulation of proteinaceous substances in the pretreatment group proved the effects of indomethacin and L-NAME on the development of nephropathy.

In renal sections of mice that were treated with iohexol, a nonionic CM, we determined an increase especially in inflammation, tubular dilatation and tubular edema and vacuolization, which was more pronounced in medium– and high-dose iohexol groups. Jeong et al. evaluated kidney sections histologically, 24 hours after the administration of 4 g of iodine/kg iohexol in mice. They determined findings of shedding, vacuole formation, loss of epithelial cell brush border and partial necrosis developed in tubular cells (2). In a study conducted in rats with non-ionic CM (2 g iodine/kg), vacuole formation was detected in the tubules at the 12th hour, but disappeared at 48th hour (18). Our findings of tubular dilatation and vacuolization were consistent with previous studies evaluating CIN at 24th hour.

In a study on mice, Rac1 was reported to be involved in the formation of myocardial superoxide and to induce atrial fibrillation by activating the NADPH oxidase complex (19). Angiotensin II and/or dietary salt intake enhance renal damage by activating mineralocorticoid receptors through the Rac1 molecule in transgenic mice injected with renin and angiotensin genes (20). In our previous study, we demonstrated that Rac1 expression and apoptosis increased in kidney tissue in a nephropathy model of streptozotocin-induced diabetic rats, and insulin treatment reduced these increases (8). In the present study however, we determined that Rac1 expression increased in all iohexol groups in comparison to the control group. The increase in Rac1 expression in the pretreatment group (Group 2) was higher than the control group (Group 1) but lower than the iohexol groups. This result suggests that Rac1 may have a role in the kidney damage of the drugs (indomethacin and L-NAME) we use to increase the severity of nephropathy. The increase in Rac1 expression in the iohexol groups in our study suggests that Rac1 may have a role in the pathogenesis of CIN, since Rac1 was shown to increase in the other nephropathies mentioned above and to induce apoptosis in many reports (8, 20).

In our study, besides Rac1 expression, we also evaluated β-PAK expression, which is the effector molecule of Rac1. We determined that β-PAK expression increased in all groups with compared to the control group, but with a statistically significant increase only in the medium– and high-dose iohexol groups. Increased Rac1 and β-PAK expressions in the pretreatment group in comparison to the control group may suggest the role of Rac1/β-PAK signal transduction pathway in the vasoconstriction induced by indomethacin and L-NAME treatment. Cellular migration was demonstrated in a study conducted using mesangial cell culture which obtained from Alport mice having L-NAME treatment-induced hypertension that it could occur via the Rac1 molecule (21). However, there are not many studies on this topic. Therefore, our study is additionally important because it demonstrates that pretreatment with L-NAME and indomethacin not only increases

apoptosis but also increases Rac1/β-PAK expressions. The fact that these two agents used in pretreatment phase of the CIN model of rodents increased Rac1/β-PAK expressions suggest the role of Rac1/β-PAK pathway in vasoconstriction and resultantly in the development of nephropathy. In this sense, our study results suggest the role of the Rac1/β-PAK pathway in conditions associated high risk of developing nephropathy (eg renal vasoconstriction, decreased renal blood flow). We determined that Rac1 expression significantly increased in kidney sections of mice treated with iohexol with respect to the control group. Interestingly, however, we observed that the increase in Rac1 expression showed a decrease as the iohexol dose increased, although it was not statistically significant. The increase in β-PAK expression however was dependent on iohexol dose. Similarly, nephropathy related findings and apoptosis rate increased dose dependently, as in the β-PAK expression. The significant increases determined in Rac1 and its major effector molecule β-PAK with respect to the control group support the possibility of the role of Rac1/β-PAK signalling pathway in iohexol-induced nephropathy. β-PAK expression and nephropathy findings tend to increase as the increased Rac1 expression decreased in the environment, which may interpreted as the increase of Rac1 activity rather than its expression as iohexol dose increased. Furthermore, the expression of Rac1 still continues to increase but the only difference now is that it does not display a dose-dependent pattern any more. However, the dose-dependent increase in effector molecule strongly supports the role of Rac1/β-PAK signalling pathway in CIN pathogenesis.

There are numerous in vitro and in vivo studies supporting the fact that CM cause nephropathy by increasing apoptosis (3, 12, 18). We also found that apoptosis increased dose dependently in all iohexol groups, which was compatible with the previous studies (12, 13). The increase we observed in the pretreatment group indicated that the agents (indomethacin and L-NAME) we used in order to induce nephropathy displayed apoptosis-enhancing effects. To the best of our knowledge, there is no study in the literature reporting that these two agents increase apoptosis in renal tissue, though, these agents were noted to increase apoptosis in other tissues. Apoptosis was shown to increase in rats having indomethacin-induced gastric ulcer, compared to the control group. L-NAME added to the protective agents in the ulcer treatment groups reduced the effectiveness of the ulcer treatment (22).

In our study, biochemical analyses of serum creatinine and cystatin C were also performed in addition to histological evaluation in order to determine the presence and severity of CIN. Creatinine analysis is a common method albeit several reports noting that it is not a good marker to assess renal function because it is affected by many factors such as muscle mass, age, and gender (12, 22). In human and animal studies conducted with a view to evaluate CIN biochemically, there are different results of serum creatinine and cystatin C analyses published in the literature due to differences in blood collection time (19, 22). Linkermann et al. (2013) developed a mouse model of CIN and reported no increase in serum creatinine and urea levels after 24 hours (20). We didn't obtain statistically significant difference among study groups in terms of serum creatinine results because we measured only at 24th hour which seem to be compatible with the data of Linkermann et al. (2013). Although serum cystatin C levels were higher in all iohexol groups than the control group, there was no statistically significant difference. High levels of cystatin C level in pretreatment group support the effect of vasoconstriction-induced decrease in renal blood flow on the development of nephropathy. For this reason, it also supports the opinion that high risk patients may be more affected by CM injection, especially in case of dehydration. The fact that the increases determined in serum cystatin C levels were more pronounced than the increases in creatinine levels invariably in all iohexol groups is consistent with many studies reporting that cystatin C is a much more sensitive marker than creatinine (19, 21). Previous studies on CIN have focused on the issue as to whether the contrast agent causes nephropathy or not, and determining the severity of nephropathy, as well as the treatments and precautions that can be effective in preventing the development of nephropathy or decreasing the risk of nephropathy.

This present study is the first study investigating the role of Rac1/β-PAK signaling pathway in the pathogenesis of CIN. In patients under risk of developing nephropathy, such as those with diabetes mellitus, CM injections may cause more pronounced damage than normal people. In our previous study, we have shown increased Rac1 expressions in the diabetic nephropathy model as well. Our results suggest that preventive therapies targeting the Rac1/β-PAK signaling pathway may be effective in patient groups such as diabetic patients, having high risks for CIN. However, further studies are needed on this subject.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - YAM, MG; Design - YAM, MG, SCM, SK; Supervision - MG, HAB; Fundings - MG, YAM; Materials - YAM, SK, AC; Data Collection and/or Processing - YAM, AC, SCM, SK; Analysis and/or Interpretation - YAM, MG; Literature Search - YAM, MG; Writing Manuscript - YAM, MG; Critical Review - MG, HAB

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This work was supported by Research Fund of the Dokuz Eylul University, project number 2013.KB.SAG.029.

Presented in: This research was presented as a poster in the 5th International Congress of Molecular Medicine, Izmir, Turkey, 2015

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