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RESEARCH ARTICLE

The Effect of N-Acetylcysteine Use on Endoplasmic Reticulum Stress in the Kidney Tissues of Obese Rats

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ABSTRACT

Endoplasmic reticulum (ER) stress has recently acquired increasing importance in the pathogenesis of obesity-associated kidney disease. N-acetylcysteine, otherwise known as NAC, is an antioxidant that works directly and indirectly by increasing the production of antioxidants in cells. A diet consisting of 60% calories from fat was used to establish the obesity model for the present investigation. In the NAC and obesity + NAC (ObNAC) groups, NAC was administered by intragastric tube at 150 mg/kg for eight weeks. GRP78 and PERK expressions were determined immunohistochemically in sections collected from kidney tissues at the end of the experiment. The GRP78 H score was significantly higher in the obese group than in the control, NAC, and ObNAC groups ($p<0.01$). The ObNAC group H-score was significantly lower than that of the obese group ($p < 0.01$) but was not different from the control and NAC groups. The obese group PERK H-score was also significantly higher than the control, NAC, and ObNAC groups ($p<0.01$). In the ObNAC group, the H-score was significantly lower than that in the obese group ($p<0.01$) and significantly higher than those in the control and NAC groups ($p<0.01$). Increasing changes in stress markers may be improved by NAC application, since obesity induced by a high-fat diet activates ER stress in kidney tissue.

Keywords: ER stress, GRP78, N-acetylcysteine, Obesity, PERK

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Obez Sıçanların Böbrek Dokularında N-Asetilsistein Kullanımının Endoplazmik Retikulum Stresi Üzerine Etkisi

ÖZ

Endoplazmik retikulum (ER) stresi son zamanlarda obezite ile ilişkili böbrek hastalığının patogenezinde artan bir önem kazanmıştır. N-asetilsistein (NAC), hücrelerde antioksidan üretimini artırarak doğrudan ve dolaylı olarak çalışan bir antioksidandır. Çalışmada, kalorinin %60'ını yağdan elde eden bir diyet ile obezite modeli oluşturuldu. NAC ve obezite + NAC (ObNAC) gruplarında NAC intragastrik tüp ile 150 mg/kg dozunda sekiz hafta süreyle uygulandı. Deney sonunda elde edilen böbrek dokularından alınan kesitlerde GRP78 ve PERK ekspresyonları immünohistokimyasal olarak belirlendi. GRP78'in H skoru obez grubunda kontrol, NAC ve ObNAC gruplarına göre anlamlı olarak yüksekti ($p<0.01$). ObNAC grubundaki H skoru, obez grubundan önemli ölçüde düşüktü ($p < 0.01$). Ayrıca bu grubun skoru kontrol ve NAC gruplarıyla benzerdi. Obez grubunda PERK H skoru kontrol, NAC ve ObNAC gruplarına göre anlamlı olarak yüksekti ($p<0.01$). ObNAC grubunda H skoru obez grubuna göre anlamlı olarak düşük ($p<0.01$), kontrol ve NAC gruplarına göre anlamlı olarak yüksekti ($p<0.01$). Yüksek yağlı diyet ile oluşan obezite böbrek dokusunda ER stresine neden olduğundan stres belirteçlerinde artan değişikliklerin NAC uygulaması ile iyileştirilebileceği düşünülebilir.

Anahtar kelimeler: ER stres, GRP78, N-asetilsistein, Obezite, PERK

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INTRODUCTION

More than 1.9 billion individuals worldwide are overweight, more than 650 million of who are clinically obese, and the numbers are rising annually. Progressively greater attention has therefore been paid to obesity-related glomerulopathy ever since obesity emerged as a widespread problem (Lu et al. 2020). High-fat diet (HFD)-induced obesity causes several complicated health issues worldwide. Human and animal research shows that obesity is linked to type 2 diabetes, cardiovascular disease, non-alcoholic fatty liver disease, and chronic kidney disease (Promsan et al. 2022). Obesity has been shown to cause both functional and histological changes in the kidneys, making them aberrant in appearance (Xu et al. 2017). Excessive HFD consumption in kidney cells can generate reactive oxygen species (ROS). Excessive levels of ROS in the kidney induce renal cell injury and dysfunction via the activation of multiple pathways, including oxidative stress, apoptosis, and impaired autophagy (Pengrattanachot et al. 2020, Promsan et al. 2022). Obesity may potentially represent a risk factor for end-stage renal disease, even after controlling for hypertension and diabetes, according to many experimental animal and human studies (Munusamy et al. 2015). Obesity is the result of a complex relationship between genetic and environmental stimuli that affect cell metabolism and homeostasis, including mitochondrial defect, dysregulated mitophagy, autophagy, and endoplasmic reticulum (ER) stress. Obesity is both a cause and an effect of an ER stress response (Ajoolabady et al. 2021).

The structure and function of the ER are complex. It plays important roles in the production, folding, changing, and transport of proteins and in the formation and distribution of phospholipids and steroids. For example, it stores calcium ions in its lumen and slowly releases these into the cytoplasm (Schwarz and Blower 2016). Any disruption in these activities will lead to ER stress and the accumulation of misfolded proteins (Zhao et al. 2018). The unfolded protein response (UPR), an adaptive mechanism, restores ER homeostasis in response to ER stress. Improved protein folding and UPR signaling increase the expression of proteins involved in the ER folding machinery. It also attenuates general protein translation, thus lightening the strain on the ER (Hetz and Papa 2018). Also known as ER-associated degradation, terminally misfolded proteins are moved from the ER to the cytoplasm and destroyed by the proteasome. Nevertheless, UPR signaling will initiate cell death pathways if the return of ER homeostasis is significantly prolonged (Adams et al. 2019, Lee and Ozcan 2014, Lin et al. 2019). The UPR, a cellular stress response originating from the ER, is controlled by three major sensors, inositol-requiring enzyme 1 (IRE1), protein kinase RNA-activated (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6). In the absence of stress, these three ER stress sensors are bound and rendered inactive by the ER-localized chaperone, also known as glucose-regulated protein 78 (GRP78/BIP), in the ER lumen. The accumulation or increase of misfolded proteins in the ER lumen activates the GRP78/BIP and the three sensors (Almanza et al. 2019).

Obesity occurs through a complex interaction of genetic and environmental factors that disrupt cellular homeostasis and metabolism. These include mitochondrial dysfunction, abnormal mitophagy, abnormal macroautophagy/autophagy, and abnormal ER homeostasis. Obesity may be both a cause and an effect of an unregulated ER stress response, according to recent research (Ajoolabady et al. 2022). Obesity may also disrupt the ER protein-folding mechanism, a phenomenon known as ER stress. GRP78, the resident ER chaperone, is disrupted as a result, thus exacerbating renal tubular damage. Experimentally induced ER stress in renal tubules and podocytes has been shown to activate autophagy and to cause cell damage (Munusamy et al. 2015). Excess free fatty acids (FFAs) accumulate in adipose tissues and ectopically in other organs, causing lipotoxicity and contributing to the development of obesity-related kidney damage. Endoplasmic reticulum stress occurs in tissues with abnormal deposits of FFA. More recent developments in lipid metabolism raise the possibility of ER stress as a common molecular mechanism in the etiology of disorders associated with hyperlipidemia (Li et al. 2019).

N-acetylcysteine (NAC) is a mucolytic agent that contains the thiol group and that is the subject of increasing research due to its potential anti-inflammatory and antioxidant effects. In addition, NAC is on the list of essential medicines maintained by the World Health Organization, and its well-established history of being safe makes it an appealing candidate for treating a diverse range of conditions. It is therefore interesting to note that a growing body of experimental data supports the therapeutic advantages of NAC therapy in managing obesity-related problems (Dludla et al. 2019). Derived from the amino acid L-cysteine, NAC is a precursor to glutathione in mammals. Studies have shown that it exhibits anti-hyperglycemic activity by protecting the β-cells of diabetic Zucker diabetic fatty rats, diabetic mice, and diabetic CD1 mice that have been alloxan-induced to become diabetic (Ho et al. 1999, Tanaka et al. 1999, Sarvani et al. 2017). Additionally, NAC reduces kidney damage induced with streptozotocin or iomeprol in mice and protects male Sprague-Dawley rats against the development of insulin resistance caused by hyperglycemia (Sarvani et al. 2017).

This study therefore investigated the expression of the ER stress indicators GRP78 and PERK in the kidney tissue of rats subjected to obesity. The potential protective effects of NAC against ER stress resulting from obesity in kidney tissue were also investigated and tested.

MATERIAL AND METHODS

Animals, Ethical Approval and Experimental Design

Sixteen 12-week-old Wistar albino female rats (250 ±50 g) were randomly assigned to each of four equal groups. The animals were allowed ad libitum access to food and water under standard conditions at 24±1 °C. Kastamonu University Animal Experiments Local Ethical Committee authorized and monitored all animal treatments (no. 2023/18). The control group received a regular diet (10% kcal) for eight weeks. The NAC group received a conventional diet (10% kcal) plus 150 mg/kg NAC (SigmaAldrich, Merck, Germany) via the intragastric route for eight weeks. The obese group received an HFD (60% kcal) (Arden Research and Experiment Company, Ankara, Turkey) for eight weeks for the induction of obesity. The obesity + NAC (ObNAC) was given an HFD (60% kcal) for eight weeks plus 150 mg/kg NAC via intragastric tube. At the end of the experiment, xylazine-ketamine (IP, 10mg/kg-50mg/kg) anesthesia was applied to examine kidney tissues after cervical dislocation. Tissues were fixed in 10% neutral buffered formaldehyde for 24 h and embedded in paraffin following routine tissue procedures.

Immunohistochemistry Procedure

Five micrometer-thick paraffin-embedded sections were placed onto poly-lysine slides and subjected to deparaffinization and rehydration. The sections were kept in sodium citrate buffer (pH=6.0) under moist heat and pressure for antigen retrieval. They were then incubated in 3% hydrogen peroxide (hydrogen peroxide 30% Merck: 108597) solution for 20 minutes and washed with PBS solution for 15 minutes. Ten percent standard goat serum blocking solution was next applied at room temperature for 10 minutes. The sections were subsequently incubated overnight at 4 °C with primary antibodies (Table 1). Following washing with PBS, the slides were incubated with a secondary antibody (TP-125-HL, Thermo Fisher Scientific, USA). The antigen-antibody complex was then demonstrated with the AEC chromogen. Gills hematoxylin was used as a counterstain, and the sections were covered. The sections were examined under a light microscope and photographed (Zeiss Axiolab 5, Jena, Germany).

Table 1. Antibodies used in immunohistochemical staining as the primary antibodies

Primary antibodies	IHC Dilution	Code	Company
GRP78/BIP Polyclonal antibody	1/200	11587-1-AP	Proteintech Group
PERK/EIF2AK3 Polyclonal antibody	1/200	24390-1-AP	Proteintech Group

Semiquantitative Scoring

GRP78 and PERK immunoreactivities were measured using a semiquantitative scoring technique based on intensity of staining (IS). This was evaluated as absence of staining (-), mild staining (++), medium staining (+++), and high staining. The average of the results from two different researchers was used to calculate the IS of immunoreactions in cells. A semiquantitative assessment of IS and the percentage of positive cells was also used to calculate the sections' immunohistochemistry scores (H-score). Finally, all antibody expression levels were compared statistically using the median H-score (Tatar et al. 2023).

Statistical Analysis

The statistical analyses were performed using the SPSS 26.0 (IBM SPSS Statistics, IBM Corporation, Chicago, IL) software for MAC. The data were analyzed for normality using the Shapiro-Wilks test. Kruskal Wallis tests were used to compare the groups. The data were presented as the median, and the interquartile range [Me (Q25–Q75)] and $p<0.05$ was recognized as statistically significant.

RESULTS

GRP78 Immunohistochemistry

GRP78 immunoreactivity was mainly localized in proximal and distal tubules and the mesangial areas of glomeruli in the obese group. This area also exhibited intense immunoreactivity compared to the other groups (Table 2). The H-score of this group was also significantly higher than those in the control, NAC, and ObNAC groups ($p<0.01$). However, in the ObNAC group, GRP78 immunoreactivity was weakly present in both the proximal tubule (PXT) and distal tubule (DT) regions but not in glomerular compartments (in either glomerular capillaries or Bowman's space) (Figure 1). This group's H-score was lower than that of the obese group ($p < 0.01$), but did not differ significantly from the control and NAC groups (Table 4).

PERK Immunohistochemistry

While no PERK immunoreactivity was detected in the control and NAC groups, very strong immunoreactivity was seen in the obese group cortex and medulla. Specifically, PERK in the obese group was localized in the proximal and distal tubules,

mesangial areas of the glomeruli, and medullary regions (Table 3). The H-score of this group was also significantly higher than those of the control, NAC, and ObNAC groups ($p<0.01$) (Table 4). In the ObNAC group, PERK immunoreactivity was moderate in the PXT, DT and medullary regions. At the same time, it was weakly expressed in the glomerular capillaries and medullar area (Figure 2). The H-score of this group was significantly lower than that of the obese group ($p<0.01$), but was also significantly higher than those of the control and NAC groups ($p<0.01$) (Table 4).

Table 2. Semiquantitative analysis of GRP78 immunoreactivity

GRP78 immunoreactivity	Groups			
Parameters	**Control**	**NAC**	**Obese**	**ObNAC**
Distal tubules	+	+	+++	+
Proximal tubules	+	+	+++	+
Glomeruli	+	+	+++	+
Medulla	-	-	-	-

-: No staining; +: weak positive; ++: moderate positive; +++: strong positive.

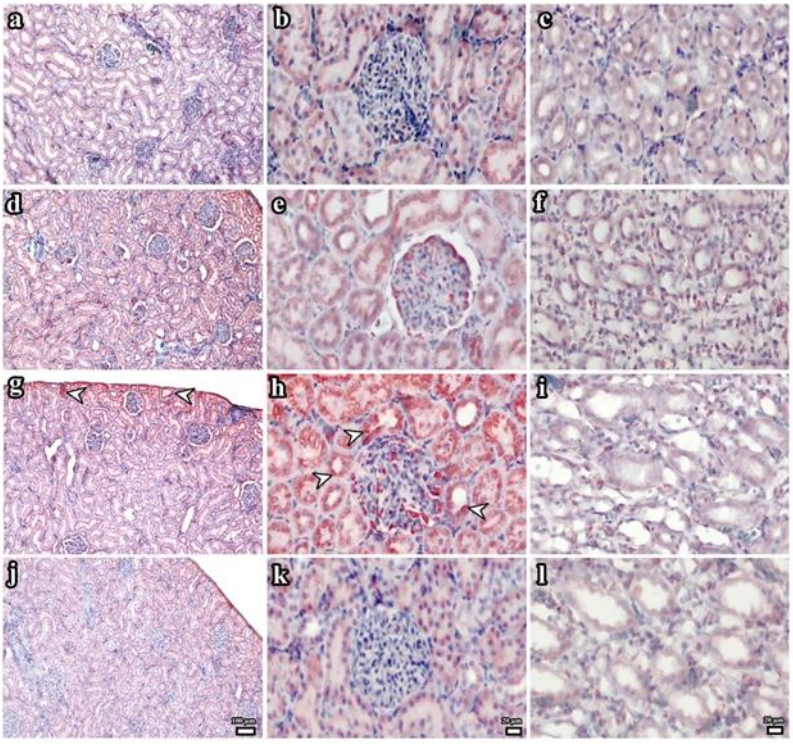

Figure 1: GRP78 expression analyses using immunohistochemistry (IHC) in kidney tissues. (a-c) Representative staining of GRP78 in the control group kidneys is weakly positive in proximal and distal tubules. (d-f) The staining of GRP78 in the NAC group is weakly positive in proximal and distal tubules and glomerular capillaries. (g-i) Densely positive staining is detected in the obese group proximal and distal tubules and glomerular capillaries. (j-l) Representative staining of GRP78 in the ObNAC group is weakly positive in the proximal and distal tubules, but with no reactivity in the glomerulus. The scale bar represents 100 μm for the left and 20 μm for the middle and right columns.

Table 3. Semiquantitative analysis of PERK immunoreactivity

PERK immunoreactivity	Groups			
Parameters	**Control**	**NAC**	**Obese**	**ObNAC**
Distal tubules	**-**	**-**	**+++**	**++**
Proximal tubules	**-**	**-**	**+++**	**++**
Glomeruli	**-**	**-**	**+++**	**++**
Medulla	**-**	**-**	**+++**	**++**

-: No staining; +: weak positive; ++: moderate positive; +++: strong positive.

Table 4. Semi-quantitative analysis of GRP78 and PERK antibody results. The data were presented as the median and the interquartile range [Me (Q25–Q75)].

	GRP78 positivity score	PERK positivity score
Control	80 (75-90) [a, d]	80 (75-100) [b]
NAC	100 (80-110) [c, d]	80 (75-110) [b]
Obese	250 (230-270) [a, b, d]	240 (200-270) [a, b, d]
ObNAC	100 (90-100) [a, b, c]	165 (150-175) [c]

[a]p = 0.000; versus the control group, [b]p = 0.000; versus the NAC group, [c]p = 0.000; versus the obese group, [d]p = 0.001; versus the ObNAC group, Kruskal–Wallis/Tamhane T2 test

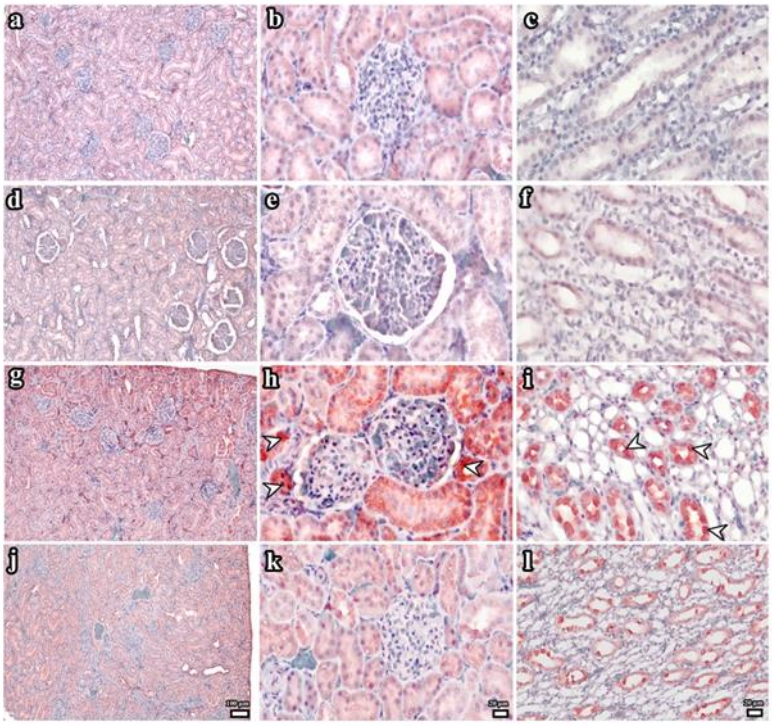

Figure 2: Analysis of PERK expression in kidney tissues by immunohistochemistry. (a-c) No immunoreactivity was detected at representative GRP78 staining in the control group. (d-f) Similarly to the control group, no GRP78 immunoreactivity is detected in the cortex or medulla in the NAC group. (g-i) In the obese group, very intense positive staining is seen in the proximal and distal tubules, and glomerular capillaries. (j-1) Representative GRP78 staining in the ObNAC group is moderately positive in the proximal and distal tubules and weak in the glomerulus and medullar region. The scale bar represents 100 μm for the left and 20 μm for the middle and right columns.

DISCUSSION

The primary purpose of this study was to investigate the hepatoprotective effect of NAC, a well-defined and safe compound, against ER stress in an obese rat model. Immunohistochemical analysis clearly showed that increased expressions of GRP78 and PERK, indicators of ER stress caused by obesity in kidney tissue, were reduced by the application of NAC. These results provide direct evidence supporting the use of NAC supplementation as an effective means of blocking obesity-related ER stress.

Despite the worldwide increase in obesity, pharmacotherapeutic alternatives for treating the disease and related disorders are still limited and inadequate. It is therefore essential to understand the molecular causes of obesity. ER stress may be used as a therapeutic target in treating the condition (Kim et al. 2013, Angelidi et al. 2022).

Obesity is a disease associated with oxidative stress and high levels of reactive oxygen species (ROS). A previous study of the association between obesity and kidney damage reported that the cafeteria diet, with its pro-oxidant effect and activation of apoptotic pathways, is capable of reducing the oxidative stress resulting from such damage (La Russa et al. 2019). Research has demonstrated that obesity generated by an HFD in several organs leads to ER stress and triggers the UPR signaling pathway (Kawasaki et al. 2012, Chen et al. 2016). ROS oxidize newly formed proteins and increase misfolded and unfolded proteins in the ER. Increases in ER stress indicators

such as GRP78, p-PERK, and p-eIF2 have been reported in mice with HFD-induced obesity (Liñares-Pose et al. 2018).

Similarly, our results also showed the presence of ER stress by revealing GRP78 activation in the kidneys of obese rats. However, although this study shows that obesity induces ER stress, we have not been able to elucidate the mechanisms involved. However, numerous studies in the literature have indicated ROS formation as the mechanism responsible for ER stress (Chong et al. 2017). In addition, ROS can act on calcium channels that are present in the ER membrane, which then increases calcium release from it. The reduced total calcium concentration in the ER lumen ultimately makes it difficult for newly produced proteins to fold correctly (Kawasaki et al. 2012, Zeeshan et al. 2016, Burgos-Morón et al. 2019, Bhattarai et al. 2021, Cui et al. 2022, Masenga et al. 2023).

A state of ER stress exists in tissues with abnormal accumulations of FFAs. ER stress is thought to represent a common molecular pathway in the pathogenesis of hyperlipidemia-related diseases (Li et al. 2019). Recent research involving obese Zucker rats, a rodent model of obesity with hypertension and metabolic syndrome, revealed kidney activation of ER stress (Wang et al. 2014). Oxidative stress and ER stress play a critical role in the pathophysiology of various kidney diseases. The renal glomeruli and tubular interstitium in both obese and diabetic animal models contain ER stress markers, suggesting a connection between chronic ER stress and kidney damage (Chen et al. 2014, Wang et al. 2014). Liu et al. (2008), determined that three hallmarks of ER-associated apoptosis, CHOP, JNK, and caspase-12, were activated in the diabetic rat kidney. Wang et al. (2014), found that the ER stress protein GRP78, eIF2α-ATF4-CHOP, caspase 12, and JNK-MAPK signaling pathways increased activation due to lipid accumulation in the kidneys of obese mice. In the same study, significant GRP78 expression was detected in both glomeruli and the tubular interstitium using the immunohistochemical method in obese control mice.

However, research has also suggested that changes in the ER membrane may also affect the activation status of IRE1 and PERK, both directly and independently of the accumulation of unfolded proteins in the ER lumen (Cherngwelling et al. 2021). The effects of ischemia-reperfusion-induced acute kidney injury worsen over time in the reperfusion phase of kidney damage. The activation of the ER stress markers GRP78 and XBP1, and CHOP expression as a result of this damage is consistent with this deleterious change (Gu et al. 2018).

Consistent with the above, our immunohistochemical results also revealed an increase in PERK activity, specifically in the kidneys of the obese group. Further studies involving ELISA and Western blotting are now needed to investigate the increase in GRP78, and PERK in kidney tissue observed in the present research. NAC has been reported to increase the rate of synthesis of the cellular antioxidant reduced glutathione (GSH) by upregulating intracellular cysteine level in several metabolic, liver, and psychiatric diseases, including obesity, in which oxidative stress and inflammation are involved (Santos et al. 2017, Tümer 2020, Tüfekci et al. 2023). NAC is a cysteine derivative with an acetyl group affixed to the nitrogen atom that can be oxidized by numerous radicals, including thiol. It also functions as a nucleophile (electron-pair donor) (Samuni et al. 2013, Elbini Dhouib et al. 2016). In addition, NAC is a precursor of reduced glutathione (Zafarullah et al. 2003). Glutathione, the cell's principal antioxidant, neutralizes reactive oxygen and nitrogen species both directly and indirectly (Dean et al. 2011). The metabolic pathways with which NAC has been found to interact include control of cell cycle and apoptosis, the development of cancer and tumors, mutagenesis, gene expression and signal transmission, immunological modulation, and mitochondrial activities (Zafarullah et al. 2003). Zhang et al. (2014), determined that NAC significantly reduced cleaved caspase 3, p53 and renal epithelial tubular cell apoptosis in a rat model of renal ischemia/reperfusion injury. Another study showed that NAC administration against hepatic ischemia-reperfusion injury significantly reduced the expression of GRP78, ATF-4, and CHOP (Sun et al. 2014). Hu et al. (2019), determined that NAC downregulates XBP1 splicing against fluoride-induced testicular apoptosis, inhibits testicular cell apoptosis by reducing IRE1α-JNK phosphorylation, and contributes to the inhibition of nuclear factor E2-related factor 2 (Nrf2)-related oxidative damage. Lee et al. (2016) also highlighted the role of NAC in ER stress-mediated diabetic nephropathy. NAC reduces levels of ER stress indicators PERK, eIF2, ATF6, GRP78, and CHOP in fatty rats. However, the molecular processes that reduce that stress are still unknown. Based on the available literature, NAC reduces oxidative stress by reducing ROS build-up and ER stress-mediated problems (Sarvani et al. 2017). HFD-derived ER stress may cause severe hepatic steatosis through the activation of the UPR pathway and the induction of apoptosis in the liver. HFD-induced hepatic steatosis and apoptosis are reversed by NAC treatment. Long-term HFD consumption by newborn offspring results in steatosis of the liver and suppresses the protective action of the UPR resulting from ER stress that leads to the hepatocyte death. NAC administration has been shown to reduce liver fat accumulation, to restore the protective effect of UPR, and to reduce hepatocyte damage and apoptosis. This antioxidant activity of NAC becomes more pronounced when it is used over an extended period (Tsai et al. 2020). Studies show that NAC exhibits a protective effect by inhibiting the apoptosis pathway associated with ER

stress (Sun et al. 2014, Lee et al. 2016). We therefore speculated that a similar protective mechanism may underlying the decrease in ER stress markers in the obese group receiving NAC in this.

CONCLUSION

The expressions of the ER stress marker chaperone GRP78 and PERK implicated in the UPR response were identified in order to investigate the mechanisms involved in kidney injury in obesity, which are currently little understood, and to show the function of NAC, a potential therapeutic target, in obesity-mediated ER stress. In the light of the results obtained, we think that NAC administration may be a useful therapeutic modality in obesity-induced ER stress. Although we did not measure ROS as an underlying mechanism of obesity-induced ER stress, other studies in the literature have considered this. ROS may therefore have triggered ER stress, which may have been ameliorated by NAC, a powerful antioxidant.

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Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' Contributions: Conceptualization, Methodology, Research, Data improvement, Visualization, Writing, Review and Editing by Musa Tatar.

Explanation: The data supporting the findings of this study available from the corresponding author, MT, upon reasonable request.

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