

Metabolic Acidosis in DKA: GPR4 as a Mediator of Cellular Dysfunction

Abstract

Acidosis, a hallmark of diabetic ketoacidosis (DKA) and other metabolic disorders, plays a central role in exacerbating cellular damage, particularly in kidney and vascular tissues. Recent studies have highlighted the proton-sensing receptor GPR4 as a critical mediator in acidosis-induced cellular dysfunction. GPR4 activation in response to low pH triggers a cascade of stress responses, including endoplasmic reticulum (ER) stress, apoptosis, and inflammatory signaling. This review synthesizes findings from multiple studies examining GPR4's role in acidosis-related renal and vascular injury. Lactate dehydrogenase (LDH) release assays and cAMP accumulation measurements revealed that GPR4 activation leads to increased cell damage and apoptosis in kidney and endothelial cells under acidic conditions. Furthermore, silencing GPR4 expression mitigated these effects, indicating that GPR4 is pivotal in driving acidosis-induced injury. In endothelial cells, GPR4 activation upregulates ER stress markers, such as CHOP and GRP78, contributing to vascular dysfunction through inflammatory responses and oxidative stress. The findings underscore the potential of targeting GPR4 in therapeutic strategies aimed at alleviating kidney and vascular complications associated with DKA and other acidotic conditions. Overall, GPR4 represents a promising molecular target for mitigating the detrimental effects of acidosis on tissue function.

Introduction

Diabetic ketoacidosis (DKA) occurs due to insulin deficiency, which forces the body to break down fat for energy (Lizzo, 2023). Through this process ketones are produced, which leads to an acidic environment within the blood. This environment leads to a condition called metabolic acidosis. Metabolic acidosis is characterized by a disturbance in the acid-base balance.

This results from a decrease in blood bicarbonate, inhibiting the buffer capabilities, which leads to an increased acidity of the blood. Metabolic acidosis leads to bicarbonate depletion, dehydration, and electrolyte imbalances worsen the condition (Gosmanov et al., 2021). The pH change also has effects on many other parts of the body. One key aspect of this includes the G protein-coupled receptor 4. This paper reviews the consequences of pH changes on downstream mechanisms of this proton-sensing GPR4 receptor. The supporting articles are interconnected in the fact that GPR4 is a critical mediator of cellular dysfunction in acidotic conditions characteristic of DKA. GPR4 is a fascinating receptor due to its dual role in sensing extracellular pH and mediating important cellular responses, including inflammation, apoptosis, and vascular dysfunction. Its relevance extends to a variety of diseases, particularly those characterized by acidosis, such as DKA, ischemic injuries, and even cancer. Understanding the exact mechanisms by which GPR4 mediates these processes is crucial for developing targeted therapies that could improve the clinical outcomes of patients suffering from acidosis-induced damage. Consequently, GPR4's role in pathophysiology and its therapeutic potential make it an exciting area of research in both basic and clinical medicine.

Apoptosis

Metabolic acidosis can lead to other issues with physiological function, specifically apoptosis in cells. In the article, “Acidosis promotes cell apoptosis through the G protein-coupled receptor 4/CCAAT/enhancer-binding protein homologous protein pathway”, the researchers aimed to examine how acidosis influences apoptosis in human renal epithelial proximal tubular epithelial cells (HK-2) and human umbilical vein endothelial (HUVEC) cells to determine the molecular pathways involved (Dong et al., 2018). They primarily focused on GPR4 signaling. Cells were transfected with GPR4 siRNA to knock down GPR4 expression. Cells were exposed

to either normal pH of 7.4 (the control) or acidic pH of 6.4. Western blot analysis was used to measure protein expression levels of GPR4 (G protein-coupled receptor 4 pH sensitive), CHOP (CCAAT/enhancer-binding protein homologous protein; a stress-related protein involved in apoptosis), and cleaved caspase-3 (a key enzyme in apoptosis). TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling) assay and LDH (lactate dehydrogenase) release assay measured cell apoptosis.

A western blot is a protein detection technique based on gel electrophoresis and antibody binding (Mahmood & Yang, 2012). It allows researchers to separate, transfer, and identify specific proteins in a sample using size and antibody specificity (Kurien & Scofield, 2006). To begin, cells or tissues are lysed to extract proteins. A protease inhibitor is added to prevent protein degradation. The protein concentration is measured for equal loading. The proteins are mixed with sodium dodecyl sulfate which denatures proteins into linear structures and coats them with a negative charge, so they migrate based on molecular weight. The sample is loaded into a polyacrylamide gel, and an electric field is applied. Smaller proteins migrate faster than larger ones. The proteins are transferred from the gel to a nitrocellulose membrane. This preserves protein positions for antibody detection. The membrane is incubated with a blocking buffer to prevent nonspecific antibody binding. A primary antibody is incubated and binds to the target protein on the membrane. A secondary antibody (linked to an enzyme horseradish peroxidase) binds to the primary antibody. A chemiluminescent substrate reacts with the enzyme, producing a visible signal. A chemiluminescence detector captures the protein bands. Band intensity is analyzed to compare protein levels. A darker band relates to a higher protein concentration. The purpose is to detect and quantify specific proteins, confirm protein expression changes, and identifies post-translational modifications (Mahmood & Yang, 2012). In the

acidosis experiment, western blot analysis was used to show that GPR4, CHOP, and cleaved caspase-3 were upregulated in acidic conditions, supporting their role in apoptosis (Dong et al., 2018).

A western blot was performed which shows how acidosis affects GPR4, CHOP, and apoptosis in the cells. The control for this experiment involved keeping the cells used in a normal pH of 7.4 and not an acidic environment of 6.4 (Dong et al., 2018). The loading control for the western blot included GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), which ensures equal protein loading, normalizes target protein expression, and verifies transfer efficiency (Dong et al., 2018). The blot shows increased GPR4, CHOP, and cleaved caspase-3 protein levels after acidosis treatment with HCl. This is shown with a darker band on each respective well when compared to the control, which shows more protein production. Acidosis increased the expression of GPR4, CHOP, and cleaved caspase-3. The protein level of GPR4 was higher in the HCl treated cells when compared to the control cells. The protein level of CHOP was higher in the HCl treated cells when compared to the control cells. The protein level of cleaved caspase-3 was higher in the HCl treated cells when compared to the control cells. The relative amount of cAMP was higher in the HCl treated cells when compared to the control cells. The significance of the data from this experiment proves how acidosis induces cell death in kidney and endothelial cells by activating GPR4, CHOP, and caspase-3, leading to apoptosis (Dong et al., 2018). Knocking down GPR4 reduces this effect, suggesting GPR4 as a potential target for treating acidosis-related kidney and vascular injuries.

The siRNA transfection experiment was performed to specifically knock down GPR4 expression in cells. siRNA (small interfering RNA) is a synthetic RNA molecule that binds to complementary mRNA sequences, leading to their degradation and preventing protein translation

(McNaughton et al., 2019). A siRNA transfection experiment is a technique used to silence gene expression by introducing small interfering RNA (siRNA) into cells. The process begins with the design and synthesis of siRNA specific to the target gene. The siRNA sequence is chosen to be complementary to a portion of the mRNA, ensuring specific binding and degradation. Once the siRNA is prepared, it is mixed with a transfection reagent (such as liposomes or polymers) that promotes entry into cells by forming complexes with the negatively charged siRNA, allowing it to pass through the cell membrane (McNaughton et al., 2019). After mixing, the siRNA-transfection reagent complex is added to cultured cells, where it is taken up through endocytosis or direct fusion with the membrane. Inside the cell, the siRNA is incorporated into the RNA-induced silencing complex (RISC), where the antisense strand of the siRNA guides the complex to bind specifically to the target mRNA (McNaughton et al., 2019). The RISC complex then cleaves the mRNA, leading to its degradation and effectively preventing the production of the target protein. To assess the success of the transfection, researchers typically perform western blot analysis or quantitative PCR (qPCR) to measure protein or mRNA levels. If the target gene is successfully silenced, its mRNA and protein expression should be significantly reduced compared to untreated or control siRNA-transfected cells (McNaughton et al., 2019). By silencing GPR4, the researchers could assess whether this receptor was necessary for acidosis-induced apoptosis. Apoptosis levels were reduced in GPR4-silenced cells compared to normal cells, suggesting that GPR4 plays a crucial role in mediating the effects of acidosis (Dong et al., 2018).

To further explore the role of GPR4 signaling, the researchers performed a measurement of cAMP levels. cAMP (cyclic adenosine monophosphate) is a secondary messenger molecule involved in various cellular signaling pathways. To measure cAMP, researchers used enzyme-

linked immunosorbent assay (ELISA) kits. Enzyme-linked immunosorbent assay (ELISA) is a widely used biochemical technique for detecting and quantifying specific proteins, antibodies, hormones, or other molecules in a sample (Alhajj et al., 2023). It works by utilizing antigen-antibody interactions combined with an enzyme-linked detection system that produces a measurable signal, usually a color change. The process begins with a capture antibody being coated onto a 96-well plate, allowing it to bind specifically to the target molecule. To prevent non-specific binding, a blocking buffer is added to the plate before introducing the sample, which may contain the target molecule (Alhajj et al., 2023). If present, the target binds to the capture antibody, and any unbound material is removed through washing. Next, a detection antibody is added, which also binds to the target molecule. This antibody may be directly linked to an enzyme (direct ELISA) or require a secondary antibody conjugated to an enzyme for detection (indirect ELISA) (Alhajj et al., 2023). After the detection antibody binds, an enzyme-specific substrate (such as tetramethylbenzidine (TMB) for horseradish peroxidase) is added, initiating a reaction that produces a color change. The intensity of this color change correlates with the concentration of the target molecule and is measured using a spectrophotometer at a specific wavelength (Alhajj et al., 2023). Since GPR4 is a G protein-coupled receptor, its activation can influence intracellular cAMP signaling. By quantifying cAMP levels under acidic conditions, the researchers determined that GPR4 activation contributes to apoptosis through this signaling pathway (Dong et al., 2018).

The TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling) assay is a method used to detect DNA fragmentation, a part of apoptosis (Kyrylkova et al., 2022). During programmed cell death, enzymes called endonucleases cleave genomic DNA into small fragments, creating free 3'-OH (hydroxyl) ends. The TUNEL assay labels these DNA breaks,

allowing researchers to identify apoptotic cells. The experiment begins with cell fixation and permeabilization to preserve cellular structures while allowing access to the DNA (Kyrylkova et al., 2022). Fixed cells or tissue sections are then incubated with terminal deoxynucleotidyl transferase (TdT), an enzyme that catalyzes the addition of labeled nucleotides (e.g., fluorescein-labeled dUTP **or** biotin-labeled dUTP) to the free 3'-OH DNA ends. This incorporation of labeled nucleotides marks fragmented DNA, enabling visualization (Kyrylkova et al., 2022). After labeling, the sample is washed to remove excess reagents. If using fluorescently labeled dUTP, apoptotic cells can be directly observed under a fluorescence microscope or analyzed by flow cytometry (Kyrylkova et al., 2022). If using biotin-labeled dUTP, an additional step is required where an enzyme-conjugated antibody (e.g., streptavidin-HRP) binds to biotin, followed by a colorimetric **or** chemiluminescent detection (Kyrylkova et al., 2022). Apoptotic cells show bright fluorescence or color staining, indicating extensive DNA fragmentation, whereas healthy cells remain unstained or faintly stained. The TUNEL assay provided critical evidence in these studies by enabling the detection of apoptotic cells through DNA fragmentation, linking acidosis, GPR4 activation, and subsequent apoptosis in various tissues, including endothelial cells and kidneys. This method allowed researchers to quantify and visualize the extent of cell death under acidotic conditions, highlighting the role of acidosis in exacerbating disease processes like DKA, kidney injury, and vascular inflammation. These findings suggest that targeting pathways such as GPR4 may offer therapeutic potential for mitigating apoptosis-related complications in diseases associated with acidosis.

Lastly, the measurement of lactate dehydrogenase (LDH) was performed to assess cytotoxicity. LDH is an enzyme found in the cytoplasm that is released into the culture medium when cell membranes are damaged. The experiment begins by collecting the cell culture

supernatant, which contains any LDH released from damaged cells (Farhana & Lappin, 2023). A colorimetric or fluorescence-based LDH assay is then performed. In the assay, the LDH enzyme catalyzes the conversion of lactate to pyruvate, generating NADH (nicotinamide adenine dinucleotide, reduced form). The newly formed NADH is then used to reduce a tetrazolium salt, producing a colored formazan product that can be quantified using a spectrophotometer at an absorbance of 490–500 nm (Farhana & Lappin, 2023). The intensity of the color is proportional to the amount of LDH released, which correlates with the extent of cell damage or cytotoxicity.

These experiments proved that acidosis increased apoptosis. Exposing the cells to an acidic environment led to higher levels of apoptosis, as evidenced by increased expression of cleaved caspase-3, a marker of cell apoptosis. The acidotic conditions upregulated the expression of G protein-coupled receptor 4 and CHOP. CHOP is known to play a significant role in endoplasmic reticulum stress-induced apoptosis. Silencing GPR4 expression in these cells resulted in decreased levels of CHOP and cleaved caspase-3, leading to reduced apoptosis under acidic conditions. These results suggest that acidosis promotes apoptosis in HK-2 cells and HUVECs (Human Umbilical Vein Endothelial Cells) by activating the GPR4/CHOP pathway. Targeting GPR4 may offer a potential therapeutic approach for conditions associated with acidosis-induced cell injury.

Endothelial cell adhesion

The role of the G protein coupled receptor 4 (GPR4) in endothelial cell adhesion under acidic conditions, provides insights into cellular responses to acidosis. In the article, “Activation of GPR4 by acidosis increases endothelial cell adhesion through the cAMP/Epac pathway”, the researchers conducted a series of in vitro experiments using human endothelial cells. Their

findings contribute to understanding diabetic ketoacidosis by highlighting how acidotic conditions influence endothelial function, potentially exacerbating vascular complications in DKA patients (Gosmanov et al., 2021). They exposed the cells to acidic conditions and measured changes in adhesion molecule expression and endothelial cell adhesion. Through gene knockdown and pharmacological inhibition, they confirmed that the cAMP/Epac pathway (Exchange protein directly activated by cAMP) was responsible for these effects. Additionally, they demonstrated that overexpression of GPR4 enhanced endothelial cell adhesion under acidic conditions, further solidifying the link between acidosis and endothelial dysfunction. These experiments help explain how acidosis in DKA might contribute to vascular complications, such as increased inflammation and permeability, by modulating endothelial cell behavior (Chen et al., 2021).

Immunofluorescence is a technique used to visualize the localization and expression of adhesion molecules in endothelial cells (Im et al., 2019). By tagging specific proteins with fluorescent antibodies, the researchers could assess whether acidic conditions increased the presence of adhesion molecules on the cell surface. This helped confirm the role of acidosis in enhancing endothelial adhesion (Chen et al., 2021). This disruption leads to compromised tissue integrity, increased inflammation, impaired repair, and activation of stress pathways, all of which contribute to the worsening of conditions such as acidosis-induced kidney and vascular injury. Understanding the mechanisms behind cell adhesion disruption in acidosis is essential for developing therapeutic strategies to mitigate these harmful effects. Endothelial cells were grown on coverslips and exposed to normal (neutral pH) or acidic conditions (low pH). Cells were then fixed and incubated with fluorescently labeled antibodies specific to adhesion molecules (e.g., ICAM-1 or VCAM-1). A fluorescence microscope was used to visualize the localization and

intensity of these proteins, allowing researchers to determine whether acidic conditions increased adhesion molecule expression on the cell surface (Chen et al., 2021).

Western blot analysis detected and quantified specific proteins, such as adhesion molecules or signaling proteins, involved in the cAMP/Epac pathway (Mahmood & Yang, 2022). By comparing protein expression levels under normal and acidic conditions, the researchers could determine whether acidosis altered protein production or activation in endothelial cells. Protein lysates were extracted from endothelial cells treated under different pH conditions. The samples were separated by gel electrophoresis and transferred to a membrane, which was incubated with antibodies targeting adhesion molecules or proteins in the cAMP/Epac signaling pathway (Chen et al., 2021). A secondary antibody conjugated to an enzyme was added, and a chemiluminescent substrate was used to visualize the protein bands. This allowed quantification of changes in protein expression due to acidosis, including increased expression of pro-inflammatory adhesion molecules such as VCAM-1 and E-selectin, as well as markers related to cell stress and apoptosis like cleaved PARP and Bax. (Chen et al., 2021).

Real time reverse transcriptase polymerase chain reaction measured the expression levels of genes related to adhesion molecules and signaling pathways (Chen et al., 2021). By analyzing mRNA levels, the study determined whether acidosis induced the transcription of genes associated with endothelial adhesion, helping link GPR4 activation to gene regulation. RNA was extracted from endothelial cells exposed to acidic and normal conditions. Reverse transcriptase was used to convert mRNA into complementary DNA (cDNA), which was then amplified using specific primers for adhesion molecules and signaling proteins (Doak & Zair, 2011). By quantifying the amplified products in real time, researchers determined whether acidosis increased the transcription of genes involved in endothelial adhesion (Chen et al., 2021).

The cAMP assay, which measured intracellular cAMP levels, was crucial for verifying GPR4 activation under acidic conditions, as GPR4 signals through the Gs pathway to elevate cAMP. This provided a functional confirmation of receptor engagement and downstream signaling in response to acidosis (Chen et al., 2021). A cAMP assay quantified cAMP production in endothelial cells exposed to acidic conditions, confirming whether GPR4 activation led to increased cAMP signaling. To measure cAMP levels, endothelial cells were incubated with acidic or normal media. After treatment, cells were lysed, and a cAMP-specific enzyme-linked immunosorbent assay (ELISA) was performed. In ELISA, a capture antibody is first coated onto a 96-well plate to bind the target molecule, followed by a blocking buffer to prevent non-specific binding (Alhajj et al., 2023). If the target is present in the sample, it binds to the capture antibody, and unbound material is washed away. A detection antibody is then added, either directly linked to an enzyme (direct ELISA) or requiring a secondary enzyme-conjugated antibody (indirect ELISA) (Alhajj et al., 2023). Finally, an enzyme-specific substrate (e.g., TMB for HRP) is added, producing a color change proportional to the target concentration, which is measured using a spectrophotometer (Alhajj et al., 2023). This helped determine whether acidosis increased cAMP production through GPR4 activation (Chen et al., 2021).

Cell adhesion Assay assessed how strongly endothelial cells adhered to a substrate or to other cells under different pH conditions (Chen et al., 2021). By quantifying cell adhesion in normal versus acidic environments, the study demonstrated that acidosis increased endothelial adhesion through GPR4 activation. Endothelial cells were plated onto a surface coated with extracellular matrix proteins or other endothelial cells and then subjected to acidic or normal conditions (Kashef & Franz, 2015). After incubation, unbound cells were washed away, and the number of adhered cells was quantified using microscopy or a colorimetric assay. Increased

adhesion under acidic conditions confirmed that acidosis promoted endothelial cell attachment (Chen et al., 2021).

RNAi knockdown was used to silence the GPR4 gene and determine its role in acidosis-induced endothelial adhesion (Chen et al., 2021). If knocking down GPR4 prevented the increase in cell adhesion under acidic conditions, it confirmed that GPR4 was essential for the observed effects. Small interfering RNA (siRNA) specific to GPR4 was transfected into endothelial cells to reduce GPR4 expression (Han, 2018). The knockdown efficiency was verified by Western blot analysis. After silencing GPR4, the cells were exposed to acidic conditions, and adhesion assays were performed. Adhesion did not increase in the knockdown cells, which confirmed that GPR4 was required for the acid-induced adhesion response (Chen et al., 2021).

Overall, this study shows that activation of GPR4 by acidosis increases endothelial cell adhesion via the cAMP/Epac signaling pathway, which could impact vascular inflammation and permeability observed in DKA (Gosmanov et al., 2021). Exposure to acidic environments led to the activation of GPR4 in endothelial cells. This mechanism may have implications for understanding vascular inflammation and related pathologies.

Vascular endothelial cell inflammatory responses

Acidosis activates the proton-sensing GPR4 receptor in vascular endothelial cells, triggering inflammatory responses (Dong et al., 2023). In the article, “Acidosis activation of the proton-sensing GPR4 receptor stimulates vascular endothelial cell inflammatory responses revealed by transcriptome analysis”, the researchers conducted transcriptome analysis using microarray technology to examine gene expression changes in endothelial cells exposed to acidic conditions. They identified upregulation of numerous inflammatory genes linked to cytokines,

adhesion molecules, and immune signaling pathways (Dong et al., 2023). Real-time RT-PCR and Western blot analysis were then used to validate the expression of key inflammatory genes and proteins. Additionally, RNAi knockdown was performed to silence GPR4, demonstrating that inflammatory gene induction was significantly reduced when GPR4 was inhibited (Dong et al., 2023). These experiments provided strong evidence that acidosis-induced inflammation in endothelial cells is mediated by GPR4, offering insights into how acidosis in DKA can contribute to vascular complications (Gosmanov et al., 2021).

Western blot analysis was used to detect and quantify specific inflammatory proteins in endothelial cells exposed to acidic conditions (Mahmood & Yang, 2022). Cells were lysed, and the extracted proteins were separated using gel electrophoresis. The proteins were then transferred to a membrane and incubated with antibodies targeting inflammatory markers such as adhesion molecules (e.g., ICAM-1, VCAM-1) or cytokines. The results showed that acidosis upregulated these proteins, confirming that GPR4 activation enhances endothelial inflammation at the protein level (Dong et al., 2023).

Real time reverse transcriptase polymerase chain reaction was used to measure changes in gene expression related to inflammation (Dong et al., 2023). RNA was extracted from endothelial cells treated under acidic or normal conditions. The RNA was converted into complementary DNA (cDNA) using reverse transcriptase, and specific inflammatory genes were amplified and quantified in real time using fluorescent probes (Doak & Zair, 2011). The results demonstrated that acidosis upregulated the transcription of pro-inflammatory genes (IL6, IL8, TNF, ICAM1, and VCAM1) supporting the findings from the transcriptome analysis (Dong et al., 2023).

cAMP assay was used since GPR4 activation is linked to intracellular signaling pathways (Dong et al., 2023). The researchers measured cyclic AMP (cAMP) levels in endothelial cells under acidic conditions. A cAMP-specific enzyme-linked immunosorbent assay (ELISA) was performed to quantify cAMP concentrations (Alhajj et al., 2023). In ELISA, a capture antibody is first coated onto a 96-well plate to bind the target molecule, followed by a blocking buffer to prevent non-specific binding (Alhajj et al., 2023). If the target is present in the sample, it binds to the capture antibody, and unbound material is washed away. A detection antibody is then added, either directly linked to an enzyme (direct ELISA) or requiring a secondary enzyme-conjugated antibody (indirect ELISA) (Alhajj et al., 2023). Finally, an enzyme-specific substrate (e.g., TMB for HRP) is added, producing a color change proportional to the target concentration, which is measured using a spectrophotometer (Alhajj et al., 2023). The assay confirmed that acidosis activated GPR4, leading to increased cAMP production, which in turn contributed to inflammatory signaling (Dong et al., 2023).

Cell adhesion assay tested how acidosis influenced endothelial cell interactions with leukocytes, a key component of inflammation (Dong et al., 2023). Endothelial cells were grown in acidic or neutral conditions and then incubated with fluorescently labeled leukocytes. After washing away unbound cells, the number of adhered leukocytes was quantified using microscopy or fluorescence detection. The increased leukocyte adhesion under acidic conditions indicated that GPR4 activation promotes endothelial inflammation by enhancing immune cell recruitment (Dong et al., 2023).

Overall, this study contributes to the understanding of diabetic ketoacidosis (DKA) by revealing that acidosis, a hallmark of DKA, can promote endothelial inflammation through GPR4 signaling (Lizzo et al., 2023). Activation of GPR4 by acidosis in HUVECs significantly

increased the expression of various inflammatory genes, including chemokines, cytokines, adhesion molecules, and genes associated with the NF- κ B pathway and prostaglandin endoperoxidase synthase 2. Further analyses indicated that the NF- κ B pathway plays a crucial role in mediating the inflammatory gene expression induced by acidosis through GPR4 activation. Acidosis-induced activation of GPR4 enhanced the adhesion of HUVECs to U937 monocytic cells under flow conditions, suggesting a potential mechanism for increased vascular inflammation. Similar upregulation of inflammatory genes was observed in other endothelial cells, such as human lung microvascular endothelial cells and pulmonary artery endothelial cells, upon GPR4 activation by acidosis. This inflammation may contribute to the vascular dysfunction observed in DKA patients, including increased endothelial permeability and leukocyte adhesion, which can exacerbate complications such as organ damage and impaired circulation (Gosmanov et al., 2021). Treatment with a GPR4 antagonist significantly reduced the endothelial inflammatory response induced by acidosis, indicating a potential therapeutic approach for conditions associated with vascular inflammation.

Renal function impairment

The role of acidosis and GPR4 in renal ischemia/reperfusion injury (Dong et al., 2024). In the article, “Ischemia/reperfusion-induced CHOP expression promotes apoptosis and impairs renal function recovery: the role of acidosis and GPR4”, the researchers aimed to examine how ischemia/reperfusion (I/R)-induced acidosis activates the proton-sensing receptor GPR4, leading to CHOP (C/EBP homologous protein) expression, apoptosis, and impaired renal function recovery (Dong et al., 2024). To explore this, the researchers conducted in vivo and in vitro models of renal ischemia/reperfusion injury to examine the effects of acidosis and GPR4 activation. Western blot analysis and RT-PCR were used to measure CHOP expression and apoptotic markers in renal tissues and cultured kidney cells under acidic conditions (Dong et al.,

2024). TUNEL staining was performed to detect apoptosis, showing increased cell death with GPR4 activation. RNAi knockdown of GPR4 demonstrated that silencing the receptor reduced CHOP expression and apoptosis, confirming its role in acidosis-induced kidney injury (Dong et al., 2024). Additionally, renal function assays assessed kidney recovery, revealing impaired function in acidotic conditions.

The renal ischemia-reperfusion (I/R) model was used to mimic kidney injury caused by interrupted and restored blood flow; a process known to create an acidic environment (Dong et al., 2024). Mice underwent surgical clamping of renal arteries to induce ischemia (lack of blood supply), followed by reperfusion (restoration of blood flow) (Shiva et al., 2020). Kidney tissues were then collected and analyzed for injury markers. Analysis was performed using the western blot method. The expression of proinflammatory cytokines, apoptosis-related proteins, and oxidative stress markers were measured using the western blot method. TUNEL assay was used to detect apoptosis by labeling fragmented DNA, which is characteristic of apoptosis. This model demonstrated how acidosis and GPR4 activation affect kidney recovery following ischemic injury, a process that contributes to renal dysfunction in DKA (Gosmanov et al., 2021).

To evaluate the severity of kidney damage, histology and histomorphological scoring of acute tubular injury were performed (Dong et al., 2024). Kidney tissue sections were stained with hematoxylin and eosin (H&E) and examined for signs of tubular damage, cell death, and structural changes. These tissue sections were examined using microscopy. A scoring system was used to quantify the extent of injury. This experiment provided histological evidence that acidosis and GPR4 activation contribute to kidney damage, supporting the hypothesis that acidosis impairs renal recovery (Dong et al., 2024).

In addition to assessing structural damage, polymorphonuclear leukocyte (PMN) infiltration was measured to evaluate the immune response in injured kidneys (Dong et al., 2024). Polymorphonuclear leukocytes (PMNs), or neutrophils, are white blood cells that play a crucial role in immune defense. They are attracted to sites of infection or injury, where they perform phagocytosis to engulf and digest pathogens. PMNs release enzymes from their granules to kill pathogens and can form neutrophil extracellular traps (NETs) to trap and eliminate microbes. Although vital for fighting infections, excessive activation of PMNs can lead to tissue damage and contribute to inflammatory diseases (Fine et al., 2020). Kidney sections were stained for PMN markers, and the infiltration of these immune cells was quantified using microscopy. The increased presence of PMNs in acidotic conditions suggested that GPR4 activation promotes an inflammatory response, contributing to kidney damage and impaired healing (Dong et al., 2024).

To directly assess apoptosis, the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay was performed (Dong et al., 2024). This technique detects DNA fragmentation, a characteristic of programmed cell death (Kyrylkova et al., 2022). Kidney tissue sections were stained with TUNEL reagents, and apoptotic cells were visualized using fluorescence microscopy. Apoptotic cells show bright fluorescence or color staining, indicating extensive DNA fragmentation, whereas healthy cells remain unstained or faintly stained. The findings demonstrated that acidosis significantly increased apoptosis in kidney cells, linking GPR4 activation to cell death and renal dysfunction (Dong et al., 2024).

In vitro studies further explored the molecular mechanisms underlying acidosis-induced kidney injury (Dong et al., 2024). Immunofluorescence staining was used to visualize the

expression and localization of CHOP and other apoptosis-related proteins in renal cells. By tagging specific proteins with fluorescent antibodies, the researchers could assess whether acidic conditions increased the expression of CHOP. This helped confirm the role of acidosis in enhancing apoptosis related proteins (Dong et al., 2024). Cells were fixed and incubated with fluorescently labeled antibodies. A fluorescence microscope was used to visualize the localization and intensity of these proteins, allowing researchers to determine whether acidic conditions increased molecule expression. The fluorescence microscopy results showed increased CHOP expression in acidotic environments, further confirming its role in GPR4-mediated apoptosis (Dong et al., 2024).

To investigate the effects of acidosis on kidney cells directly, cell culture and treatment experiments were conducted (Dong et al., 2024). Cultured renal cells were exposed to acidic media to mimic the acidic environment observed in conditions like diabetic ketoacidosis (DKA). To examine the role of G protein-coupled receptor 4 (GPR4) in acidotic stress, some cells were treated with GPR4 inhibitors, while others underwent siRNA-mediated knockdown to reduce GPR4 expression. Following treatment, various cellular responses were analyzed to assess the impact of acidosis and GPR4 activation. Western blot analysis was used to evaluate changes in apoptosis-related proteins (e.g., Bax, Bcl-2, and cleaved caspase-3) and inflammatory markers (TNF- α , IL-6). Additionally, TUNEL staining was conducted to detect DNA fragmentation as an indicator of apoptosis. These experiments provided insights into the molecular mechanisms by which GPR4 signaling contributes to acidosis-induced kidney cell injury, helping to differentiate whether the observed effects were GPR4-dependent or caused by general acidotic stress (Lizzo et al., 2023).

The measurement of lactate dehydrogenase (LDH) release was performed to assess cell membrane damage and cytotoxicity (Dong et al., 2024). LDH, an enzyme released when cells experience membrane damage, was measured in culture media using a colorimetric assay. The experiment measures LDH release from damaged cells by collecting the cell culture supernatant (Farhana & Lappin, 2023). A colorimetric or fluorescence-based LDH assay is performed, where LDH catalyzes the conversion of lactate to pyruvate, generating NADH. NADH then reduces a tetrazolium salt, producing a colored formazan product that is measured using a spectrophotometer at 490–500 nm. The color intensity directly correlates with LDH levels, indicating the extent of cell damage or cytotoxicity. The results indicated that LDH release was significantly higher in acidotic conditions, suggesting that GPR4 activation contributes to cell injury and death (Dong et al., 2024).

To determine whether GPR4 directly regulates CHOP expression and apoptosis, siRNA interference was used to silence GPR4 expression in kidney cells (Dong et al., 2024). siRNA transfection is a technique used to silence gene expression by introducing small interfering RNA (siRNA) into cells (McNaughton et al., 2019). The experiment begins with designing siRNA that is complementary to the target mRNA, ensuring specific binding and degradation. The siRNA is then mixed with a transfection reagent (e.g., liposomes or polymers) to facilitate its entry into cells. Once inside, siRNA is incorporated into the RNA-induced silencing complex (RISC), where it guides the degradation of the target mRNA, preventing protein translation. To confirm gene silencing, western blot analysis is performed to measure reductions in mRNA and protein levels compared to controls (McNaughton et al., 2019). After exposure to acidic conditions, CHOP levels and apoptosis markers were analyzed through western blot. The reduction in CHOP expression and apoptosis after GPR4 knockdown confirmed that GPR4 plays a critical role in

acidosis-induced kidney injury (Dong et al., 2024). Western blot analysis was employed to measure protein levels of CHOP, apoptotic markers, and inflammatory proteins (Dong et al., 2024). Proteins were extracted from kidney cells and tissue samples, separated via SDS-PAGE, and transferred onto membranes. These membranes were then incubated with antibodies targeting specific proteins of interest, followed by detection through chemiluminescence. The results demonstrated that acidosis and GPR4 activation upregulated CHOP expression and apoptotic signaling pathways, reinforcing the study's findings (Dong et al., 2024).

To assess the involvement of intracellular signaling pathways, measurement of intracellular cyclic AMP (cAMP) accumulation was performed (Dong et al., 2024). Renal cells were treated with acidic media, and cAMP levels were quantified using an enzyme-linked immunosorbent assay (ELISA). In ELISA, a capture antibody is first coated onto a 96-well plate to bind the target molecule, followed by a blocking buffer to prevent non-specific binding (Alhajj et al., 2023). If the target is present in the sample, it binds to the capture antibody, and unbound material is washed away. A detection antibody is then added, either directly linked to an enzyme (direct ELISA) or requiring a secondary enzyme-conjugated antibody (indirect ELISA) (Alhajj et al., 2023). Finally, an enzyme-specific substrate (e.g., TMB for HRP) is added, producing a color change proportional to the target concentration, which is measured using a spectrophotometer (Alhajj et al., 2023). Increased cAMP levels under acidosis confirmed that GPR4 activation modulates intracellular signaling pathways involved in inflammation and apoptosis, providing a potential target for therapeutic intervention (Dong et al., 2024).

These experiments provided strong evidence that acidosis-mediated GPR4 activation promotes CHOP expression and apoptosis, contributing to renal dysfunction, which may also be

relevant in DKA-related kidney injury (Gosmanov et al., 2021). This study contributes to the understanding of diabetic ketoacidosis (DKA) by demonstrating that acidosis, a key feature of DKA, exacerbates kidney injury through GPR4 activation and subsequent apoptotic signaling (Lizzo et al., 2023). Since kidney dysfunction is a complication of severe DKA, these findings highlight a potential mechanism by which acidosis worsens renal outcomes in affected patients (Gosmanov et al., 2021). Renal ischemia-reperfusion injury (IRI) significantly increased CHOP expression, which was closely associated with heightened apoptosis in kidney tissues. Mice lacking CHOP (CHOP^{-/-}) showed markedly reduced kidney injury and apoptosis compared to wild-type mice, indicating that CHOP plays a detrimental role in renal IRI. Further investigation revealed that this protective effect stemmed from CHOP deficiency in renal parenchymal cells rather than inflammatory cells, as demonstrated through bone marrow transplantation experiments. Both endothelial and epithelial cells in the kidney contributed to CHOP upregulation following IRI, promoting apoptosis and impeding functional recovery. Importantly, hypoxia-induced acidosis was identified as a key driver of CHOP expression in endothelial cells, and neutralizing this acidic environment led to reduced CHOP levels and decreased cell death. The study also demonstrated that GPR4, a proton-sensing receptor, mediates this acidosis-induced CHOP expression, as knocking down GPR4 significantly lowered CHOP levels and apoptosis in endothelial cells under hypoxia-reoxygenation conditions. These findings suggest that CHOP and GPR4 are critical mediators in acidosis-driven kidney injury and targeting either may represent a potential therapeutic strategy for improving outcomes following renal IRI.

Endoplasmic reticulum stress pathways

Acidosis activates the endoplasmic reticulum stress pathways through the proton-sensing receptor GPR4 in human vascular endothelial cells (Dong et al., 2022). In the article, “Acidosis

activates endoplasmic reticulum stress pathways through GPR4 in human vascular endothelial cells”, the researchers conducted cell culture experiments, where human vascular endothelial cells were exposed to acidic conditions to assess changes in ER stress markers (Dong et al., 2022). Western blot analysis was performed to detect the expression of ER stress-related proteins, such as CHOP and GRP78, confirming their upregulation under acidosis (Dong et al., 2022). Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was used to measure the mRNA expression of genes involved in ER stress and inflammation. Additionally, RNA interference (RNAi) knockdown of GPR4 was employed to determine whether GPR4 activation was responsible for the observed ER stress response. Cells with silenced GPR4 exhibited reduced ER stress marker expression, confirming the receptor’s role in this pathway (Dong et al., 2022).

Cell culture and retroviral transduction were used to study the effects of acidosis on endothelial cells and to manipulate GPR4 expression (Dong et al., 2022). Human vascular endothelial cells were cultured under controlled conditions and transduced with retroviral vectors to either overexpress or silence GPR4. Endothelial cells are transduced with retroviral vectors to introduce or silence specific genes for stable expression. The process begins with the production of retroviral particles, where a retroviral plasmid carrying the gene of interest is transfected into packaging cells along with helper plasmids encoding viral proteins (Liao et al., 2017). The infectious viral supernatant is then collected and used to infect endothelial cells, with enhancers like polybrene or spinoculation to improve efficiency. After transduction, antibiotic selection is applied to isolate successfully infected cells, ensuring long-term gene expression or knockdown. The effectiveness of transduction is confirmed using western blot analysis. Researchers then assess functional outcomes, such as cell viability, apoptosis, and stress marker expression, to

study the effects of genetic modifications, such as GPR4 regulation in acidosis. This approach allowed the researchers to assess the specific role of GPR4 in mediating the ER stress response under acidic conditions (Dong et al., 2022). By comparing cells with normal GPR4 expression to those with reduced or increased GPR4 activity, they determined that this receptor was responsible for the observed cellular stress responses (Dong et al., 2022).

To simulate acidic conditions, isocapnic and hypercapnic pH treatments were applied to cultured endothelial cells (Dong et al., 2022). In isocapnic acidosis, the extracellular pH was lowered while maintaining normal carbon dioxide (CO₂) levels, mimicking metabolic acidosis as seen in DKA (Dong et al., 2022). In contrast, hypercapnic acidosis involved increased CO₂ levels, modeling respiratory acidosis (Dong et al., 2022). To implement these conditions, endothelial cells were cultured in buffered media, where pH was carefully adjusted using acidic reagents such as hydrochloric acid (HCl) or sodium bicarbonate (NaHCO₃) while monitoring CO₂ levels with a controlled gas chamber or incubator. Cells were exposed to these conditions for a defined period, and their responses were analyzed using western blot to assess ER stress markers (CHOP, GRP78, ATF4) and inflammatory cytokines. By differentiating between these conditions, the researchers could examine whether the ER stress response was specifically triggered by extracellular pH changes or CO₂-related effects. The results confirmed that acidosis alone, rather than CO₂ fluctuations, activated ER stress pathways through GPR4 (Dong et al., 2022).

Western blotting was performed to assess the expression of key endoplasmic reticulum (ER) stress markers, including CHOP and GRP78, in endothelial cells exposed to acidic conditions (Dong et al., 2022). The experiment began with protein extraction, where cellular

proteins were collected using lysis buffers containing protease and phosphatase inhibitors to preserve protein integrity. The extracted proteins were then quantified to ensure equal loading across samples before being separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) based on their molecular weight. After electrophoresis, the proteins were transferred onto a nitrocellulose or PVDF membrane, where they were blocked with a solution (e.g., BSA or milk) to prevent non-specific binding. The membranes were then incubated with primary antibodies specific to CHOP and GRP78, followed by HRP-conjugated secondary antibodies that enable detection. Using chemiluminescent substrates, the researchers visualized protein bands and quantified their intensity using imaging software. By comparing protein expression levels under different pH conditions, researchers determined whether acidosis upregulated ER stress pathways. Increased expression of CHOP and GRP78 in acid-treated cells provided strong evidence that acidosis induces ER stress in endothelial cells. These findings helped establish a link between acidotic stress and endothelial dysfunction, further supporting the role of GPR4-mediated signaling in the cellular stress response (Dong et al., 2022).

To complement protein analysis, reverse transcription polymerase chain reaction was used to measure the mRNA expression of ER stress-related genes (Dong et al., 2022). In RT-PCR, RNA was extracted from endothelial cells, reverse-transcribed into complementary DNA (cDNA), and amplified to detect specific gene transcripts. This provided a quantitative measurement of these gene expression changes, confirming that acidosis significantly increased the transcription of ER stress markers (Dong et al., 2022). This experiment reinforced the findings from western blot analysis, demonstrating that acidosis-induced GPR4 activation regulates ER stress at both the transcriptional and protein levels (Dong et al., 2022).

This study contributes to the understanding of DKA by demonstrating that acidosis-induced ER stress in endothelial cells can lead to inflammation, oxidative stress, and vascular dysfunction, which are critical complications in severe DKA cases (Dong et al., 2022). Acidic conditions significantly upregulate endoplasmic reticulum (ER) stress-related genes in human umbilical vein endothelial cells (HUVECs), including CHOP (C/EBP homologous protein) and ATF3 (activating transcription factor 3). This ER stress response was shown to be mediated through the proton-sensing receptor GPR4, highlighting its critical role in detecting extracellular pH changes and initiating intracellular stress signaling. These results indicate that acidosis can activate ER stress pathways via GPR4 in vascular endothelial cells, potentially contributing to vascular dysfunction in acidic tissue environments. By linking acidosis to ER stress activation, the paper highlights a potential molecular mechanism underlying endothelial damage in acidotic conditions. Collectively, these experiments provided strong evidence that acidosis-induced GPR4 activation leads to ER stress in endothelial cells, a mechanism that may contribute to vascular complications seen in DKA (Dong et al., 2022).

Conclusion

In conclusion, the studies reviewed provide compelling evidence that acidosis, a hallmark of various pathophysiological conditions such as diabetic ketoacidosis (DKA), plays a central role in exacerbating cellular damage through the activation of the GPR4 receptor. These findings emphasize the intricate mechanisms by which acidosis influences cellular responses, particularly in endothelial and renal cells, which are crucial for vascular and kidney function. Acidosis-induced GPR4 activation triggers a cascade of biological events, including increased apoptosis, endoplasmic reticulum (ER) stress, and inflammatory responses, which contribute to the dysfunction of various tissues.

A key insight from these studies is the activation of the GPR4 receptor in response to acidic conditions, which leads to the upregulation of stress-related genes such as CHOP/CCAAT/enhancer binding proteins and ATF3 in endothelial cells. This ER stress response, mediated through GPR4 signaling, plays a pivotal role in endothelial damage and vascular dysfunction, which are critical complications in DKA and other acidotic conditions. Notably, increased expression of CHOP has been linked to both apoptotic cell death and the promotion of inflammation, highlighting its dual role in mediating cellular injury. In renal cells, acidosis-driven GPR4 activation and subsequent CHOP upregulation have been shown to significantly contribute to kidney dysfunction, particularly in the context of ischemia-reperfusion injury (IRI). The observation that mice lacking CHOP exhibit reduced kidney injury and apoptosis underscores the importance of CHOP in driving renal damage under acidic conditions.

Furthermore, the studies demonstrate that acidosis-induced inflammation, mediated by GPR4 activation, leads to increased endothelial cell adhesion, oxidative stress, and permeability, which exacerbate vascular complications such as leukocyte infiltration and organ damage. This inflammatory response is particularly relevant in the context of DKA, where acidosis contributes to the dysfunction of both the vasculature and kidneys, complicating the clinical management of these patients. The upregulation of inflammatory genes through the NF- κ B pathway, as seen in endothelial cells exposed to acidic environments, further underscores the systemic effects of acidosis on vascular health.

Importantly, therapeutic strategies targeting GPR4 or its downstream signaling pathways, such as the GPR4/CHOP axis, may offer promising interventions for alleviating the detrimental effects of acidosis in conditions like DKA. Studies have shown that the use of GPR4 antagonists

can significantly reduce the inflammatory response and improve endothelial function under acidic conditions, suggesting that blocking this receptor could be a potential therapeutic approach for mitigating the vascular and renal complications associated with acidosis.

Collectively, these findings highlight the critical role of acidosis in driving cellular dysfunction, inflammation, and apoptosis through the GPR4-mediated pathways. By elucidating the molecular mechanisms involved, these studies provide valuable insights into the pathogenesis of acidosis-related complications and underscore the potential of GPR4 as a therapeutic target for improving outcomes in patients with DKA and other acidotic conditions. Further research into the precise molecular interactions between acidosis, GPR4, and downstream signaling pathways will be essential for the development of more targeted and effective treatments for these challenging diseases.

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