Original Paper

The Proton-Activated Receptor GPR4 Modulates Glucose Homeostasis by Increasing Insulin Sensitivity

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Key Words

GPR4 • Proton-sensing receptor • Insulin • Glucose metabolism

Abstract

Background: The proton-activated G protein-coupled receptor GPR4 is expressed in many tissues including white adipose tissue. GPR4 is activated by extracellular protons in the physiological pH range (i.e. pH 7.7 - 6.8) and is coupled to the production of cAMP. **Methods:** We examined mice lacking GPR4 and examined glucose tolerance and insulin sensitivity in young and aged mice as well as in mice fed with a high fat diet. Expression profiles of pro- and anti-inflammatory cytokines in white adipose tissue, liver and skeletal muscle was assessed. **Results:** Here we show that mice lacking GPR4 have an improved intraperitoneal glucose tolerance test and increased insulin sensitivity. Insulin levels were comparable but leptin levels were increased in GPR4 KO mice. *Gpr4*^{-/-} showed altered expression of PPARα, IL-6, IL-10, TNFα, and TGF-1β in skeletal muscle, white adipose tissue, and liver. High fat diet abolished the differences in glucose tolerance and insulin sensitivity between *Gpr4*^{+/+} and *Gpr4*^{-/-} mice. In contrast, in aged mice (12 months old), the positive effect of GPR4 deficiency on glucose tolerance and insulin sensitivity was maintained. Liver and adipose tissue showed no major differences in the mRNA expression of pro- and anti-inflammatory factors between aged mice of both genotypes. **Conclusion:** Thus, GPR4 deficiency improves glucose tolerance and insulin sensitivity. The effect may involve an altered balance between pro- and anti-inflammatory factors in insulin target tissues.

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Introduction

Insulin is a major regulator of systemic glucose homeostasis by stimulating glucose uptake and utilization by insulin-sensitive tissues such as liver, skeletal muscle, and adipose tissue as well as by regulating gluconeogenesis and glycogenolysis. Insulin resistance and type II diabetes can develop or are further promoted by pro-inflammatory cytokines, adipokines, or oxidative stress associated with conditions such as obesity or ageing [1-3]. Metabolic acidosis, as found in patients with chronic kidney disease, can further contribute to the development or maintenance of insulin resistance [4-7]. Similarly, lower blood bicarbonate levels are associated with insulin resistance and a higher risk to develop type 2 diabetes [8, 9]. The mechanisms by which acidosis affects insulin sensitivity and contributes to the development of type 2 diabetes have not been elucidated to date.

In 2003 the G-protein-coupled receptors OGR1 (Ovarian cancer G-protein-coupled receptor 1, GPCR68), GPR4, and TDAG8 (T-cell death associated gene 8, GPCR65) were recognized to be activated by extracellular protons [10, 11]. These receptors had been initially described as receptors activated by sphingosylphosphorylcholine (SPC), lysophosphatidylcholine (LPC) and modulated by psychosine [11]. However, these publications were subsequently retracted [12-14]. OGR1 and GPR4 are widely expressed on mRNA level in most organs, whereas the expression of TDAG8 appears to be restricted to few organs such as immune cells and bone. All three receptors are activated by extracellular protons in the physiological range for most extracellular fluids, e.g. very low activity for pH more alkaline than pH 7.8 and full activity around pH 6.8 [10, 11, 15]. GPR4 and TDAG8 are coupled to the production of cAMP whereas OGR1 is predominantly linked to phospholipase C activity, intracellular calcium rises, and protein kinase C stimulation [10, 11, 15-17].

The physiological function of these receptors is not fully understood to date. Mice lacking *Ogr1* show impaired insulin secretion by pancreatic Beta cells but have normal glucose tolerance [18]. Also GPR4 deficient mice have been generated and described to suffer from a form of renal acidosis [19]. GPR4 and TDAG8 have been linked to the regulation of inflammatory cytokines and factors and chemotactic migration of inflammatory cells [20-25]. Therefore, we were interested to test if GPR4 modulates glucose homeostasis and if this effect would be associated with changes in the profile of pro- and anti-inflammatory factors in insulin target organs.

Here we demonstrate that GPR4 deficient mice have improved glucose tolerance due to increased insulin sensitivity. This phenotype is maintained in aged mice but not in mice with diet induced obesity and is associated with differential regulation of several pro- and anti-inflammatory factors in muscle, liver, and white adipose tissue.

Materials and Methods

Animals

All experiments were performed with wild-type (*Gpr4*^{+/+}) and *Gpr4* deficient (*Gpr4*^{-/-}) mice bred in a pure Balb/c background. The generation, breeding and genotyping of *Gpr4* KO mice have been described previously [26].

Mice were maintained on a normal chow diet (4.5% fat, KLIBA NAFAG) if not stated otherwise. Some animals were fed a high fat diet (45% fat, Research Diets) beginning at 12 weeks of age. In each experiment, age-matched wild-type littermate mice were used as controls.

All experiments were performed according to the Swiss Animal Welfare laws and approved by the local veterinary authority (Veterinäramt Zürich).

mRNA extraction and semi-quantitative RT-PCR

Tissues were harvested and rapidly frozen in liquid nitrogen. Snap-frozen tissues were homogenized in RLT-Buffer (Qiagen, Basel, Switzerland) supplemented with β -mercaptoethanol to a final concentration of 1%. Total RNA was extracted from 200 μ l aliquots of each homogenized sample using the RNeasy Mini

Table 1. List of primers and probes for semi-quantitative real-time RT-PCR

Gene	Primers	Probe	Acc. No.
CD-11c	S: 5'-TAC TGA GTT CAT CAT TCA AGC AGA G (456-480) A: 5'-GGA ACA CGA TGT CTT GGT CTT GCT (516-539)	5'-ACT TCC CAA CTG CAC AGC AGG AGT GTC (486-512)	NM_021334
IL-10	S: 5'-CCT TAA TGC AGG ACT TTA AGG GTT A (273-297) A: 5'-GGG CAT CAC TTC TAC CAG GTA AAA (332-355)	5'-TGG GTT GCC AAG CCT TAT CGG AAA TG (300-325)	NM_010548
IL-1β	S: 5'-AAT GGA CAG AAT ATC AAC CAA CAA G (532-556) A: 5'-CTG CAG GGT GGG TGT GCC GTC T (660-681)	5'-TGA GCT TTG TAC AAG GAG AAC CAA GCA AC (569-597)	NM_008361
IL-6	S: 5'-AAC AAT CTG AAA CTT CCA GAG ATA C (305-329) A: 5'-GCT ATG GTA CTC CAG AAG ACC AGA (392-415)	5'-TGA TGG ATG CTA CCA AAC TGG ATA TAA TC (337-365)	NM_031168
PPAR-α	S: 5'-GAT TCA GAA GAA GAA CCG GAA CA (877-899) A: 5'-TGC TTT TTC AGA TCT TGG CAT TC (969-991)	5'-ATG TCA CAC AAT GCA ATT CGC TTT GG (941-966)	NM_011144
TGF-1β	S: 5'-AGA GGT CAC CCG CGT GCT A (632-650) A: 5'-GCT TCC CGA ATG TCT GAC GTA (722-742)	5'-ACC GCA ACA ACG CCA TCT ATG AGA AAA CC (658-686)	NM_011577
TNFα	S: 5'-CAG ACC CTC ACA CTC AGA TCA TCT (393-416) A: 5'-CCT CCA CTT GGT GGT TTG CT (455-474)	5'-ATT CGA GTG ACA AGC CTG TAG CCC ACG T (424-451)	NM_013693

Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Quality and concentration of the isolated RNA preparations were analyzed on a ND-1000 spectrophotometer (NanoDrop Technologies). Total RNA samples were stored at -80°C. Each RNA sample was diluted to 100 ng/μl and 3 μl used as a template for reverse transcription using the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For reverse transcription, 300 ng of RNA template were diluted in a 40-μl reaction mix that contained (final concentrations) RT buffer (1x), MgCl₂ (5.5 mM), random hexamers (2.5 μM), RNase inhibitor (0.4 U/μl), the multiscribe reverse transcriptase enzyme (1.25 U/μl), dNTP mix (500 μM each), and RNase-free water.

Semi-quantitative real-time qRT-PCR was performed on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Carlsbad, California). Primers for all genes of interest were designed using Primer Express software from Applied Biosystems primers (see Table 1). Primers were chosen to result in amplicons no longer than 150 bp spanning intron-exon boundaries to exclude genomic DNA contamination [27]. The specificity of all primers was first tested on mRNA derived from liver and white adipose tissue and always resulted in a single product of the expected size (data not shown). Probes were labelled with the reporter dye FAM at the 5' end and the quencher dye TAMRA at the 3' end (Microsynth, Balgach, Switzerland). Real-Time PCR reactions were performed using TaqMan Universal PCR Master Mix (Applied Biosystems). Briefly, 3.5 μl cDNA, 1 μl of each primer (25 μM), 0.5 μl labelled probe (5 μM), 6.5 μl RNase free water, 12.5 μl TaqMan Universal PCR Master Mix reached 25 μl of final reaction volume. Reaction conditions were: denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/elongation at 60°C for 60 seconds with auto ramp time. All reactions were run in duplicate. For analysing the data, the threshold was set to 0.06 as this value had been determined to be in the linear range of the amplification curves for all mRNAs in all experimental runs. The expression of gene of interest was calculated in relation to GAPDH or hypoxanthine guanine phosphoribosyl transferase (HPRT) as indicated. Relative expression ratios were calculated as $R=2^{(Ct(HPRT)-Ct(test\ gene))}$, where Ct represents the cycle number at the threshold 0.06.

Glucose tolerance and insulin sensitivity tests

Mice were fasted overnight (from 6 p.m. to 8 a.m. next day) with free access to water. Body weight and blood glucose were measured, and animals injected with either glucose (2 g/Kg body weight) or insulin (0.75 IU/g BW) i.p. For measuring glucose tolerance, glucose measurements were repeated after 30, 60, 120, and 150 min. To determine insulin sensitivity, glucose measurements were repeated 15, 30, 45, and 60 min after insulin injection. After the last glucose measurement, mice received a bolus of glucose to facilitate recovery. Blood for glucose measurements was obtained by puncturing the tail vein, and glucose levels were measured using Accu-Chek Aviva (Roche Diagnostics, Basel).

Measurements of insulin and leptin

Plasma samples from 5 WT and 5 KO mice were prepared, immediately frozen and shipped to Rules Based Medicine (<http://www.myriadrbm.com/products-services/the-rbm-approach/>) for measurement of analytes.

Fig. 1. Expression of GPR4 mRNA in various mouse tissues. Semi-quantitative real-time RT-PCR was performed on various mouse tissues (n = 5 male mice) and GPR4 mRNA detected in most organs. Highest relative mRNA levels were seen in abdominal white adipose tissue.

Fig. 2. Glucose homeostasis in 10 weeks old *Gpr4*^{+/+} and *Gpr4*^{-/-} mice. (A) Blood glucose levels in male mice eating ad libitum (n = 5 *Gpr4*^{+/+} and 7 *Gpr4*^{-/-} mice). (B) Intraperitoneal glucose tolerance test in overnight fasted male *Gpr4*^{+/+} (n = 10) and *Gpr4*^{-/-} (n = 9) mice. (C) Insulin sensitivity test in overnight fasted male *Gpr4*^{+/+} (n = 8) and *Gpr4*^{-/-} mice (n = 9). Data are shown as mean ± SEM, * p ≤ 0.05, ** p ≤ 0.01.

Statistics

All data were tested for statistical significance using the unpaired t-test and p < 0.05 was considered as statistically significant.

Results

Expression of GPR4 in white adipose tissue

GPR4 is widely expressed in mouse tissues as indicated by detection of GPR4 mRNA using semi-quantitative RT-PCR (Fig. 1). Among the tissues with high levels of GPR4 mRNA abundance are spleen, lung, and white adipose tissue.

Fig. 3. Leptin and insulin levels. Blood insulin and leptin levels were measured in *Gpr4^{+/+}* (n = 5) and *Gpr4^{-/-}* (n = 5) mice. Data are shown as mean $\pm$ SEM, * p $\leq$ 0.05.

Absence of GPR4 increases insulin sensitivity

Blood glucose levels were measured in the morning (9 a.m.) in 10 weeks-old male animals with free access to water and food overnight. Wild-type mice had blood glucose levels significantly higher than their *Gpr4^{-/-}* littermates (218.2 ± 14.1 mg/dl vs. 162.9 ± 13.6 mg/dl, $p < 0.05$, $n = 5$ *Gpr4^{+/+}* and 7 *Gpr4^{-/-}*) suggesting that GPR4 may modulate glucose homeostasis (Fig. 2A). Metabolic cage experiments had indicated similar rates of food intake (0.14 g/g BW/24 hrs for *Gpr4^{+/+}* and 0.14 g/g BW/24 hrs for *Gpr4^{-/-}*, respectively, $n = 6$ per group). In order to directly test glucose tolerance, male mice were fasted overnight (with free access to water) and injected intraperitoneally the next morning with glucose (2 g/ Kg BW) and blood glucose was monitored for 150 min after injection. *Gpr4^{-/-}* had lower initial blood glucose levels, however, after glucose injection *Gpr4^{-/-}* showed increased blood glucose levels to similar values as *Gpr4^{+/+}* mice but returned faster to baseline values than their wild-type littermates (Fig. 2B, $n = 10$ *Gpr4^{+/+}* and $n = 9$ *Gpr4^{-/-}*). Female mice were also tested and showed a similarly improved intraperitoneal glucose tolerance test (data not shown). Next we tested the effect of injecting insulin (0.75 IU/g BW) into male mice after overnight fasting. Fasting glucose levels were lower in *Gpr4^{-/-}* but this difference did not reach significance in this particular set of experiments. Insulin caused a faster and stronger fall in blood glucose levels in *Gpr4^{-/-}* than in *Gpr4^{+/+}* mice demonstrating increased insulin sensitivity in the absence of GPR4 (Fig. 2C, $n=8$ *Gpr4^{+/+}* and 9 *Gpr4^{-/-}*). Again, female mice showed similar results (data not shown). Bodyweight was similar between wild-type and KO mice if corrected for gender. We also dissected mice and measured weights of organs, total fat, and skeleton plus muscle and did not find any difference between genotypes (6 animals each) (data not shown).

Next, we measured plasma leptin and insulin levels in mice fed ad libitum and found no difference for insulin (*Gpr4^{+/+}* 0.85 ± 0.09 μ IU vs. *Gpr4^{-/-}* 1.09 ± 0.14 μ IU, $n = 5$ each) but higher levels of leptin in GPR4 KO mice (*Gpr4^{+/+}* 0.42 ± 0.02 ng/ml vs. *Gpr4^{-/-}* 0.62 ± 0.07 ng/ml, $n = 5$ each).

Altered expression of pro- and anti-inflammatory factors in insulin target tissues

The major insulin-sensitive tissues are liver, skeletal muscle and white adipose tissue and insulin sensitivity and glucose uptake are modulated by a variety of factors including pro- and anti-inflammatory factors. We examined therefore the expression of major modulators of insulin sensitivity in these tissues. In skeletal muscle lower expression of IL-6 and PPAR α were found whereas TNF α , IL-10, IL-1 β , CD-11c, and TGF-1 β were not altered (Fig. 4A). In liver, PPAR α was reduced and IL-10 expression increased. All other factors remained unchanged (Fig. 4B). The strongest changes were found in white adipose tissue with TNF α and TGF-1 β being elevated and IL-6 and PPAR α being reduced (Fig. 4C). Taken together, these data suggest that absence of GPR4 alters the balance between pro- anti-inflammatory factors in insulin target tissues.

Fig. 4. mRNA expression of pro- and anti-inflammatory factors in young mice. Semi-quantitative RT-PCR was performed on tissues from young (10 weeks old) *Gpr4*^{+/+} and *Gpr4*^{-/-} mice and mRNA expression of pro- and anti-inflammatory factors assessed. (A) mRNA expression in skeletal muscle (upper hind leg), (B) in liver, and (C) in abdominal white adipose tissue. n = 5 *Gpr4*^{+/+} and n = 5 *Gpr4*^{-/-}, * p ≤ 0.05, ** p ≤ 0.01.

High fat diet and ageing modulate the impact of GPR4 on glucose metabolism

Obesity due to high fat diet or ageing negatively impacts on glucose metabolism and insulin sensitivity [1-3]. Thus, we tested if obesity and ageing modulated the positive effect of GPR4 deletion on insulin sensitivity.

In a first series, we fed 12 week old *Gpr4*^{+/+} and *Gpr4*^{-/-} mice for 16 weeks with a high fat diet (45% fat). Body weight gain was identical in both groups (data not shown). We also monitored intraperitoneal glucose tolerance (IPGTT) before the start of the diet (12 weeks old mice) and at the end of the diet (28 weeks old mice). As expected, in 12 weeks old mice, IPGTT was improved and insulin sensitivity increased in *Gpr4*^{-/-} mice (Fig. 5A,C). After 16 weeks of high fat diet *Gpr4*^{+/+} showed in the IPGTT elevated glucose levels as compared to the IPGTT before the start of the diet consistent with reduced glucose tolerance (Fig. 5B). In *Gpr4*^{-/-} the increase in blood glucose levels was even more pronounced. Insulin sensitivity was similar in *Gpr4*^{+/+} and *Gpr4*^{-/-} (Fig. 5D). Consistently, we also found higher glucose levels in *Gpr4*^{-/-} fed ad libitum whereas fasting blood glucose levels were similar in both genotypes (Fig. 5E).

Therefore, we examined expression of pro- and anti-inflammatory factors in white adipose tissue and liver collected from mice either fed or fasted overnight. In mice fed ad libitum, elevated expression of TNF α and TGF-1 β was found in white adipose tissue. In the same tissue, higher levels of IL-6 and lower levels of PPAR α mRNA was found in fasting *Gpr4*^{-/-} mice (Fig. 6A). In liver tissue, only IL-10 mRNA was increased in fed *Gpr4*^{-/-} mice. In fasting animals, IL-6 and PPAR α were reduced in *Gpr4*^{-/-} liver whereas IL-10 and CD-11c were highly elevated (Fig. 6B).

Fig. 5. High fat diet abolishes improved glucose tolerance and higher insulin sensitivity in *Gpr4*^{-/-} mice. *Gpr4*^{+/+} and *Gpr4*^{-/-} received high fat diet for 16 weeks. (A) Intraperitoneal glucose tolerance test (IPGTT) in *Gpr4*^{+/+} and *Gpr4*^{-/-} mice at the age of 12 weeks before the start of the high fat diet. (B) Insulin tolerance test in *Gpr4*^{+/+} and *Gpr4*^{-/-} mice at the age of 12 weeks before the start of the high fat diet. (C) Intraperitoneal glucose tolerance test (IPGTT) in *Gpr4*^{+/+} and *Gpr4*^{-/-} mice at the age of 28 weeks after the high fat diet. (D) Insulin tolerance test in *Gpr4*^{+/+} and *Gpr4*^{-/-} mice at the same age. (E) Blood glucose levels in fasted and fed *Gpr4*^{+/+} and *Gpr4*^{-/-} mice at the age of 28 weeks. $n = 18$ *Gpr4*^{+/+} and $n = 10$ *Gpr4*^{-/-}. Data are shown as mean $\pm$ SEM, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

In a second series of animals, we allowed mice to age to 12 months ($n = 9$ per genotype) and assessed IPGTT and insulin sensitivity. In contrast to mice fed a high fat diet, *Gpr4*^{-/-} retained an improved IPGTT and greater insulin sensitivity than wild-type littermates (Fig. 7). Measurement of mRNAs of pro- and anti-inflammatory factors in white adipose tissue and liver did not detect any significant differences between *Gpr4*^{+/+} and *Gpr4*^{-/-} mice, respectively (Fig. 8A and 8B).

Fig. 6. mRNA expression of pro- and anti-inflammatory factors in mice after 16 weeks of high fat diet. Semi-quantitative RT-PCR was performed on tissues from *Gpr4*^{+/+} and *Gpr4*^{-/-} mice receiving a high fat diet for 16 weeks and mRNA expression of pro- and anti-inflammatory factors assessed. (A) mRNA expression in abdominal white adipose and (B) in liver. n = 6 *Gpr4*^{+/+} and n = 7 *Gpr4*^{-/-}, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

Fig. 7. Maintained improved glucose tolerance and higher insulin sensitivity in aged *Gpr4*^{-/-} mice. *Gpr4*^{+/+} and *Gpr4*^{-/-} at the age of 12 months were tested by (A) an intraperitoneal glucose tolerance test (IPGTT) with (B) an insulin tolerance test. Data are shown as mean ± SEM, ** p ≤ 0.01, *** p ≤ 0.001.

Discussion

We present three major findings in this study: i) GPR4 modulates glucose tolerance by increasing the sensitivity to insulin, ii) the effect of GPR4 is lost in obese animals but not in aged animals, and iii) the absence of GPR4 alters the expression profile of pro- and anti-inflammatory factors in insulin target tissues.

In the absence of GPR4, mice had lower fasting and fed glucose levels. Fed mice had also lower insulin and higher leptin levels. The intraperitoneal glucose tolerance test confirmed the improved glucose tolerance in *Gpr4*^{-/-} and the insulin sensitivity test demonstrated that the improved glucose tolerance is most likely due to enhanced insulin sensitivity. The lower insulin levels in fed mice are consistent with this notion. This is in contrast to mice lacking another member of the pH-sensitive G protein-coupled receptors, OGR1 [18]. OGR1 as well as GPR4 and TDAG8 are expressed in pancreatic β -cells and OGR1 modulates their insulin secretion in response to glucose or KCl induced depolarization coupled to a pathway involving intracellular Ca^{2+} rises [18]. The role of GPR4 in β -cells requires further experiments but our data suggest that the improved glucose tolerance is at least in part explained by higher insulin sensitivity of insulin target organs. In mice lacking TDAG8 we did not find any change in glucose tolerance suggesting that TDAG8 might be involved in other functions in these cells (data not shown).

We tested next if the improved glucose tolerance and insulin sensitivity in *Gpr4* KO mice was maintained if mice were challenged with a high fat diet for 16 weeks or if aged,

Fig. 8. mRNA expression of pro- and anti-inflammatory factors in 12 month old mice. Semi-quantitative RT-PCR was performed on tissues from *Gpr4*^{+/+} and *Gpr4*^{-/-} mice aged 12 months and mRNA expression of pro- and anti-inflammatory factors assessed. (A) mRNA expression in abdominal white adipose and (B) in liver. n = 6 *Gpr4*^{+/+} and n = 6 *Gpr4*^{-/-}, * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

conditions associated with increased insulin resistance. High fat diet caused a similar increase in body weight in both groups, abolished the difference in insulin sensitivity, and caused an even higher glucose intolerance in *Gpr4*^{-/-} mice. In contrast, in aged mice the higher insulin sensitivity and improved glucose tolerance were maintained in mice lacking GPR4. Thus, absence of GPR4 does not per se improve insulin sensitivity but the effect is modulated by other factors.

Deletion of GPR4 was associated with profound changes in the mRNA expression profile of various pro- and anti-inflammatory factors in insulin target organs that have been shown to affect glucose tolerance and insulin sensitivity. We tested the pro-inflammatory molecules TNF α , Cd11c, a marker for pro-inflammatory M1 macrophages, IL-6, and IL-1 β which cause reduced glucose tolerance and insulin resistance by acting on pancreatic β -cells as well as on the pathways mediating cellular insulin effects such as IRS-1 phosphorylation [28-32]. In young GPR4 deficient mice, reduced levels of IL-6 were found in muscle and white adipose tissue, whereas TNF α was up-regulated in white adipose tissue. When GPR4 KO mice were challenged with a high fat diet or aged, reduced IL-6 levels persisted in liver but were elevated in white adipose tissue from acutely fed GPR4 KO mice. TNF α was reduced in white adipose tissue of fasted GPR4 KO mice.

We also assessed the mRNA expression of anti-inflammatory markers in the same tissues, namely PPAR α , IL-10 and TGF1 β which have been shown to improve glucose tolerance and/or insulin sensitivity [1, 31, 33]. Surprisingly, in GPR4 KO mice PPAR α was down-regulated in most conditions in liver, white adipose tissue and skeletal muscle. In contrast, profound increases were observed for IL-10 in liver from young mice as well as from mice fed a high fat diet. TGF1 β showed only weak regulation in abdominal white fat tissue being slightly upregulated in young *Gpr4*^{-/-} mice which would support increased insulin sensitivity in this tissue. However, in aged mice maintaining improved insulin sensitivity, no evidence for TGF1 β regulation was detected suggesting that increased insulin sensitivity may occur independent from TGF1 β regulation.

Despite strong regulation of pro- and anti-inflammatory markers in GPR4 KO mice, it remains unclear if and how these changes caused improved glucose tolerance and insulin sensitivity. Factors involved in modulating glucose tolerance and insulin sensitivity showed patterns of regulation that did not clearly correlate with improved glucose tolerance of young or aged GPR4 KO. In the case of the high fat fed mice, GPR4 KO showed an even reduced glucose tolerance, however, IL-10 associated with improved glucose tolerance was up-regulated. In aged mice, no significant regulation of any of the factors tested was found despite highly improved glucose tolerance in GPR4 deficient mice. Thus, either other factors not identified to date mediate the effects of GPR4 deletion on insulin sensitivity or the balance between the pro- and anti-inflammatory factors is more strongly influenced by single factors than obvious from our data.

Related to this problem is the question, in which organ and cell type(s) and how GPR4 modulates insulin sensitivity. GPR4 mRNA was detected in several major organs involved in insulin-sensitive glucose metabolism, most prominently in white adipose tissue. However, this tissue consists of several cell types including adipocytes, immune cells, and vascular cells. The localization of GPR4 on cellular level has been hampered by the fact that no specific antibodies have been available up to date and that alternative methods such as in-situ hybridization failed to detect GPR4 mRNA reliably. Recent reports, however, indicate that GPR4 is highly expressed in endothelial cells [26, 34] and that endothelial cells play a critical role in modulating insulin sensitivity of adipose tissue [35].

GPR4 deficient mice are mildly acidotic [19] and acidosis has been associated with increased insulin resistance and reduced glucose tolerance in CKD patients [4-7]. Whether GPR4 participates in this dysregulation being more activated in acidosis and thereby reducing insulin sensitivity remains to be examined.

In summary, we demonstrate that the proton-activated G protein-coupled receptor GPR4 modulates glucose tolerance and insulin sensitivity in mice and this effect is maintained in aged mice but not in animals fed a high fat diet.

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