Stimulation of glucose uptake in murine soleus muscle and adipocytes by 5-(4-phenoxybutoxy)psoralen (PAP-1) may be mediated by Kv1.5 rather than Kv1.3

Kv1 channels are shaker-related potassium channels that influence insulin sensitivity. Kv1.3 $^{-/-}$ mice are protected from diet-induced insulin resistance and some studies suggest that Kv1.3 inhibitors provide similar protection. However, it is unclear whether blockade of Kv1.3 in adipocytes or skeletal muscle increases glucose uptake. There is no evidence that the related channel Kv1.5 has any influence on insulin sensitivity and its expression in adipose tissue has not been reported. PAP-1 is a selective inhibitor of Kv1.3, with 23-fold, 32-fold and 125-fold lower potencies as an inhibitor of Kv1.5, Kv1.1 and Kv1.2 respectively. Soleus muscles from wild-type and genetically obese *ob/ob* mice were incubated with 2-deoxy[1-^{14}C]-glucose for 45 min and formation of 2-deoxy[1-^{14}C]-glucose-6-phosphate was measured. White adipocytes were incubated with D-[U-^{14}C]-glucose for 1h. TNFalpha and Il-6 secretion from white adipose tissue pieces were measured by enzyme-linked-immunoassay. In the absence of insulin, a high concentration (3 µM) of PAP-1 stimulated 2-deoxy[1-14C]-glucose uptake in soleus muscle of wild-type and obese mice by 30% and 40% respectively, and in adipocytes by 20% and 50% respectively. PAP-1 also stimulated glucose uptake by adipocytes at the lower concentration of 1 µM, but at 300 nM, which is still 150-fold higher than its EC50 value for inhibition of the Kv1.3 channel, it had no effect. In the presence of insulin, PAP-1 (3 µM) had a significant effect only in adipocytes from obese mice. PAP-1 (3 µM) reduced the secretion of TNFalpha by adipose tissue but had no effect on the secretion of IL-6. Expression of Kv1.1, Kv1.2, Kv1.3 and Kv1.5 was determined by RT-PCR. Kv1.3 and Kv1.5 mRNA were detected in liver, gastrocnemius muscle, soleus muscle and white adipose tissue from wild-type and ob/ob mice, except that Kv1.3 could not be detected in gastrocnemius muscle, nor

Kv1.5 in liver, of wild-type mice. Expression of both genes was generally higher in liver and muscle of *ob/ob* mice compared to wild-type mice. Kv1.5 appeared to be expressed more highly than Kv1.3 in soleus muscle, adipose tissue and adipocytes of wild-type mice. Expression of Kv1.2 appeared to be similar to that of Kv1.3 in soleus muscle and adipose tissue, but Kv1.2 was undetectable in adipocytes. Kv1.1 could not be detected in soleus muscle, adipose tissue or adipocytes. We conclude that inhibition of Kv1 channels by PAP-1 stimulates glucose uptake by adipocytes and soleus muscle of wild-type and ob/ob mice, and reduces the secretion of TNFalpha by adipose tissue. However, these effects are more likely due to inhibition of Kv1.5 than to inhibition of Kv1.3 channels.

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INTRODUCTION

Kv1 channels are *shaker*-related, voltage-gated potassium channels (Gutman *et al.*, 2003). It has been proposed that compounds that block the Kv1.3 channel might be used in the treatment of type 2 diabetes and obesity (Choi & Hahn, 2010). Thus, a variant of the human Kv1.3 gene is associated with low insulin sensitivity and impaired glucose tolerance (Tschritter *et al.*, 2006), whereas Kv1.3$^{-/-}$ mice were protected from diet-induced insulin resistance (Xu *et al.*, 2003) and obesity (Xu *et al.*, 2004). Some studies on inhibitors of Kv1.3 have demonstrated their ability to improve insulin sensitivity in mice (Xu *et al.*, 2004, Upadhyay *et al.*, 2013), but another has failed to show any benefit (Straub *et al.*, 2011).

In those studies in which improved insulin sensitivity has been shown, the tissues where the inhibitors have their primary action are uncertain. Kv1.3 channels in liver, postganglionic sympathetic neurons and brown adipose tissue are suggested targets (Upadhyay *et al.*, 2013). It is unclear whether white adipose tissue or skeletal muscle could be the primary site of action because there is disagreement as to whether Kv1.3 is expressed, particularly at the protein level, in these tissues (Grande *et al.*, 2003, Li *et al.*, 2006, Straub *et al.*, 2011, Upadhyay *et al.*, 2013). Nevertheless, the Kv1.3 inhibitor margatoxin decreased TNFα secretion by white adipose tissue from genetically obese (*ob/ob*) mice (Xu *et al.*, 2004), and administration of the highly selective Kv1.3 inhibitor ShK-186 for 45 days to mice fed on an obesity-inducing diet reduced TNFα mRNA in visceral adipose tissue (Upadhyay *et al.*, 2013). The target for the latter effect might be Kv1.3 channels in inflammatory cells, such as macrophages. Decreased inflammation of adipose tissue, including decreased secretion of TNFα, would be expected to improve insulin sensitivity (Calle & Fernandez, 2012).

32   As part of an investigation into the potential of Kv1.3 inhibitors for the treatment of type 2

33   diabetes, we have studied the effect of 5-(4-phenoxybutoxy)psoralen (PAP-1) on glucose uptake

34   in white adipocytes and soleus muscle, and TNFα secretion by white adipocytes from wild-type

35   and *ob/ob* mice. PAP-1 is a selective inhibitor of Kv1.3, being at least 23-fold selective as an

36   inhibitor of Kv1.3 over other Kv1-family channels and 500-fold selective over Kv2.1, Kv3.1,

37   Kv3.2 and Kv4.2 channels (Schmitz *et al.*, 2005). We report that a concentration of PAP-1 that is

38   not selective for Kv1.3 stimulated glucose uptake and reduced TNFα secretion, but a lower

39   concentration was ineffective, in agreement with the results of others (Beeton *et al.*, 2006, Straub

40   *et al.*, 2011). This raised the question of the target for the non-selective concentration of PAP-1

41   and led us to investigate the expression of Kv1.1, Kv1.2, Kv1.3 and Kv1.5 in mouse skeletal

42   muscle and white adipose tissue. Kv1.5 is identified as a candidate mediator of the effects of

43   PAP-1 on glucose uptake.

MATERIALS AND METHODS

Materials

All materials, including PAP-1 (>98% purity), were obtained from Sigma-Aldrich, Poole, UK, unless otherwise stated.

Animals

Housing and procedures were conducted in accordance with the UK Government Animal (Scientific procedures) Act 1986 and approved by the University of Buckingham Ethical review Board. C57Bl/6 and *ob/ob* mice (Harlan, Bicester, UK), aged 5-6 weeks, were fed standard laboratory chow *ad libitum* and euthanized 3-4 hours after the onset of day light cycle, by a UK Government Animal Scientific Act 1986 schedule 1 method.

RT-PCR

Tissues isolated from wild-type and *ob/ob* female C57Bl/6 mice were homogenized in Tri-reagent using a ribolyser and total RNA prepared using Qiagen^{TM} minicolumns according to the manufacturer's instructions. One μg total RNA was reverse-transcribed using avian reverse transcriptase and random priming in a 50 μl reaction. Two μl cDNA was subsequently used per 50 μl PCR reaction as standard. GAPDH was chosen as the housekeeping genes because it showed consistent C_T values in adipose tissue, adipocytes and soleus muscle. Gene expression assays were obtained from Applied Biosystems Assay-on-Demand predesigned and optimized assays.

Subsequently, tissues were isolated from wild-type female C57Bl/6 mice. Total RNA was extracted and RT PCR performed by Real Time PCR using optimized Assay-on-Demand (Applied Biosystems) primers and probes for Kv1.1, 1.2, 1.3 and 1.5, relative to an endogenous

66   GAPDH control ([Wargent *et al.*, 2013](#)). Expression of each potassium channel was calculated

67   relative to expression of GAPDH in each sample and then the expression levels of Kv1.2 and

68   Kv1.5 were expressed relative to Kv1.3 in the same tissue (Wargent et al 2013). Multiple Kv

69   channels were not run in the same PCR reaction in order to prevent competition between the

70   cDNA templates for the amplification reactants and to optimize reaction efficiency.

71   **2-Deoxyglucose uptake by soleus muscle**

72   Uptake of 2-deoxyglucose by the soleus muscle was measured by a method that we have

73   described previously ([Ngala *et al.*, 2008](#)). Soleus muscles were dissected from both hind legs of

74   wild-type and *ob/ob* C57Bl/6 male mice. Distal and proximal tendons were tied under resting

75   tension to stainless steel clips and pre-incubated for 60 min in Krebs-Henseleit bicarbonate buffer

76   that contained 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5.5 mM

77   glucose and 0.14% (w/v) fatty acid-free bovine serum albumin (BSA) at pH 7.4 and 37°C. The

78   buffer had been gassed previously with 95% O_2: 5% CO_2. They were then incubated for 45 min in

79   fresh medium that also contained 2-deoxy[1-^{14}C] glucose (56 mCi mmol^{-1}; 0.1 μCi ml^{-1}) and

80   glucose (5.5 mM), with or without insulin (10 nM for wild-type mice and 100 nM for *ob/ob*

81   mice) and PAP-1 (3 μM) or a combination of insulin and PAP-1.

82   At the end of the incubation, the muscles were digested with 1 M NaOH and the digest was

83   neutralised with 1 M HCl. One portion of the mixture was treated with 2.5 volumes of 6% (w/v)

84   perchloric acid to precipitate out proteins, leaving supernatant that contained both 2-

85   deoxyglucose and 2-deoxyglucose-6-phosphate. Another portion was treated with 2.5 volumes of

86   2.68% (w/v) Ba(OH)$_2$ and 2.51% (w/v) ZnSO$_4$ to precipitate out 2-deoxyglucose-6-phosphate.

87   Radioactivity incorporated into 2-deoxyglucose-6-phosphate was obtained from the difference

88   between that in the two supernatants. 2-Deoxyglucose uptake was calculated by dividing

89   radioactivity in 2-deoxyglucose-6-phosphate by the specific activity of 2-deoxyglucose.

90   **Glucose uptake by adipocytes**

91   Adipocytes were prepared from the parametrial fat pads of wild-type and *ob/ob* female mice by a

92   method that we have described previously (Zaibi *et al.*, 2010). Tissue was minced and digested

93   with collagenase type II in Krebs-Ringer HEPES buffer containing 10 mM HEPES, 1% bovine

94   serum albumin (fraction V), 2.5 mM $CaCl_2$, 5.5 mM glucose and 200 nM adenosine at pH 7.4 and

95   37°C. It was filtered through 250–300 μm nylon mesh. The infranatant was removed and the

96   floating layer of adipocytes was washed four times with a fresh buffer. Adipocytes were

97   concentrated to 40% of final volume of Krebs-Ringer HEPES buffer containing 5% BSA and 0.3

98   mM glucose and pre-incubated for 45 min under 95% O_2: 5% CO_2 before dispensing them into

99   300 μl polyethylene tubes for measurement of glucose uptake.

100 Glucose transport was measured as described previously (Kashiwagi, Huecksteadt & Foley,

101 1983). Adipocytes were incubated in Krebs-Ringer HEPES buffer containing BSA, 0.3 mM

102 glucose and D-[U-^{14}C] glucose (0.2 μmol/l; 0.2 μCi/ml), for 1 h at 37°C in the absence or

103 presence of different concentrations of PAP-1 and insulin. The reaction was stopped by

104 separation of the cells through silicone oil and radioactivity in the cells was measured.

105 Extracellular space was measured in parallel incubations using D-[U-^{14}C] sucrose. Secretion is

106 expressed per mg protein, determined using a method based on the Lowry assay (Bio-Rad, Hemel

107 Hempstead, UK) or per g cells.

108 **TNFα and IL-6 secretion**

109 Adipose tissue from *ob/ob* and wild-type female mice was minced into pieces weighing about

110 200 mg and added to 0.3 ml Dulbecco's modified Eagle's medium and Ham's F-12 in a 1:1

111   mixture (both from Invitrogen, Paisley, UK), supplemented with 0.5% (w/v) endotoxin-free

112   BSA . The incubation medium also contained 1 nM T3 and 5 mM glutamine. The plates were

113   incubated with or without PAP-1 (3 μM) for 30 min. TNFα and IL-6 in the culture medium was

114   assayed using murine ELISA kits (from Diaclone, Besançon, France for TNF; from Invitrogen for

115   IL-6) , following the manufacturer's instructions. Secretion is expressed per mg protein,

116   determined using the Lowry assay.

117   **Statistical analysis**

118   Glucose uptake was calculated assuming that 2-deoxyglucose and glucose are not distinguished

119   by uptake mechanisms. Data were analyzed by one- or two-way ANOVA followed by Fisher's

120   least significant difference test, as described in the figure legends, using GraphPad Prism version

121   5 (GraphPad software, San Diego CA, USA). Results are expressed as means ± SEM.

122   **RESULTS**

123   **Glucose uptake in soleus muscle**

124   Insulin (10 nM) and PAP-1 (3 μM) stimulated 2-deoxyglucose uptake in soleus muscle from both

125   wild-type and *ob/ob* mice (Fig. 1). The combination of insulin and PAP-1 also stimulated uptake

126   compared to baseline in muscle from both wild-type and *ob/ob* mice ($P<0.001$), but it did not

127   have a significantly greater effect than either insulin or PAP-1 alone.

128   **Glucose uptake in Adipocytes**

129   PAP-1 (3 μM) stimulated glucose in adipocytes from both wild-type and obese mice in the

130   absence of insulin. In the presence of insulin at a range of concentrations, its effect was

131   significant only in adipocytes from *ob/ob* mice (Fig 2). In the absence of PAP-1, insulin

132   stimulated uptake at concentrations of 0.5 nM and higher. The combination of insulin and PAP-1

133   stimulated uptake compared to baseline, but insulin did not stimulate glucose uptake significantly

134   in the presence of PAP-1. A concentration-response curve conducted using adipocytes from wild-

135   type mice in the absence of insulin showed significant effects of PAP-1 at 1 and 3 μM, but not at

136   lower concentrations (Fig.3).

137   **TNFα and IL-6 secretion**

138   PAP-1 (3 μM) suppressed the secretion of TNFα by 50% in adipocytes from both wild-type and

139   *ob/ob* mice (Fig.4). PAP-1 did not affect IL-6 secretion (results not shown). Surprisingly,

140   secretion of TNFα from *ob/ob* adipocytes was lower than from wild-type adipocytes. Others

141   have found a higher concentration of TNFα in adipocytes from *ob/ob* compared to wild-type

142   adipocytes (Galvez, San Martin & Rodriguez, 2009).

143   **Expression of Kv1mRNA**

144   Using reverse transcription PCR, Kv1.3 mRNA was detected in liver, gastrocnemius muscle,

145   soleus muscle and parametrial white adipose tissue of female wild-type and *ob/ob* mice, except

146   that expression was not significantly above baseline in gastrocnemius muscle from wild-type

147   mice (Fig.5A). Expression was higher in liver, gastrocnemius muscle and soleus muscle, but not

148   adipose tissue, of *ob/ob* mice compared to wild-type mice. Kv1.5 mRNA was detected in liver,

149   gastrocnemius muscle, soleus muscle and parametrial white adipose tissue of female wild-type

150   and *ob/ob* mice, except that expression could not be detected in livers from wild-type mice (Fig.

151   5B). Expression was higher in liver and soleus muscle of *ob/ob* mice compared to wild-type

152   mice.

153   After it was found that PAP-1 did not stimulate glucose uptake by adipocytes at concentrations

154   that should be sufficient to activate Kv1.3, Real-Time PCR was used to detect mRNA for Kv1.1,

155   Kv1.2, Kv1.3 and Kv1.5 in each of adipose tissue, adipocytes and soleus muscle. Assuming PCR

156 reaction efficiencies of 1.0 for each gene, Kv1.5 was 30-, 15- and 78-fold more highly expressed

157 than Kv1.3 in adipose tissue, adipocytes and soleus muscle respectively. Kv1.2 mRNA could not

158 be detected in adipocytes and appeared to have lower expression than Kv1.3 in adipose tissue

159 (Fig.6). Kv1.1 could not be detected in adipose tissue, adipocytes or soleus muscle.

DISCUSSION

161   A variety of evidence suggests that inhibition of Kv1.3 potassium channels might be effective in

162   the treatment of insulin resistance, and thereby of type 2 diabetes ([Choi & Hahn, 2010](#), [Upadhyay](#)

163   [et al., 2013](#)). This includes the finding that margatoxin, which inhibits members of the Kv1

164   family, including Kv1.3, lowered blood glucose in wild-type but not Kv1.3$^{-/-}$ mice ([Xu et al.,](#)

165   [2004](#)). The authors linked this finding to the demonstration that margatoxin inhibited the

166   secretion of TNFα and IL-6 by isolated adipocytes in adipose tissue and skeletal muscle after it

167   was administered to wild-type but not Kv1.3$^{-/-}$ mice. The tissues that contain the Kv1.3 channels

168   at which the inhibitors exert their initial effects have not been well-defined, however.

169   We investigated whether the initial effects of Kv1.3 inhibitors might be on Kv1.3 channels in

170   adipocytes or skeletal muscle using PAP-1. PAP-1 inhibits Kv1.3 with 33-fold selectivity over

171   Kv1.1, 125-fold selectivity over Kv1.2 and 23-fold selectivity over Kv1.5 ([Schmitz et al., 2005](#)).

172   Inhibition of K channels might be expected to lead to cell depolarization and an increased

173   demand for energy. We found that PAP-1 at the high concentration of 3 μM stimulated glucose

174   uptake in the absence of insulin in isolated soleus muscle and adipocytes from both wild-type and

175   *ob/ob* mice. PAP-1 also stimulated glucose uptake in the presence of a range of concentrations of

176   insulin in adipocytes from *ob/ob* mice. The same concentration of PAP-1 (3 μM) also reduced

177   the secretion of TNFα by adipocytes, potentially consistent with the suggestion of others ([Xu et](#)

178   [al., 2004](#)) that this is a mechanism by which blockade of Kv1.3 improves insulin sensitivity.

179   However, when we investigated the concentration-response curve for the effect of PAP-1 on

180   glucose uptake, we found that, whilst 1 μM was effective, a concentration of 300 nM, which is

181   still 150-fold higher than its EC_{50} value for blockade of murine Kv1.3 ([Schmitz et al., 2005](#)), had

182   no effect on glucose uptake.

183 These experiments were conducted using tissues from female mice, except that soleus muscles

184 from male mice were used for studying glucose uptake. These choices were in line with our

185 previous work, allowing more accurate quality control by comparison with our historic data.

186 Most studies of the effects of Kv1.3 inhibitors on metabolism have been conducted using male

187 mice, but two studies have shown that deletion of the Kv1.3 gene has similar metabolic effects in

188 male and female mice (Xu et al., 2003, Tucker, Overton & Fadool, 2008).

189 Our results are consistent with a report that PAP-1 failed to lower blood glucose or enhance the

190 glucose lowering effect of insulin when administered as a single dose or over five days to *ob/ob*

191 or *db/db* mice in amounts that gave blood levels that would selectively block Kv1.3. Moreover,

192 10 nM PAP-1, a concentration fivefold higher than its IC_{50} value for inhibition of Kv1.3 channels,

193 did not alter K^+ currents in a human skeletal muscle cell line or glucose uptake in mouse 3T3-L1

194 adipocytes (Straub et al., 2011). Similarly, margatoxin did not stimulate glucose utilization by rat

195 epididymal or mesenteric adipocytes (Beeton et al., 2006) when used at concentrations (1 or 100

196 nM) well above its EC_{50} value (30 pM) for inhibition of Kv1.3 (Garcia-Calvo et al., 1993).

197 Upadhyay et al. (Upadhyay et al., 2013) have reported that the highly selective Kv1.3 inhibitor

198 ShK-186 reduces obesity and hyperglycemia in mice fed on an obesogenic diet, but they too rule

199 out an involvement of white adipocyte or skeletal muscle Kv1.3 channels, mainly because ShK-

200 186 did not stimulate glucose uptake by white adipose tissue or skeletal muscle. ShK-186

201 doubled glucose uptake in brown adipose tissue, a site of high glucose uptake and uncoupled

202 oxidative phosphorylation, making this a more likely explanation for the anti-obesity and anti-

203 hyperglycemic effects of ShK-186, in their view. The same group had previously reported that

204 ShK-186 also failed to stimulate glucose uptake by rat white adipocytes (Beeton et al., 2006).

205 Although we do not believe that PAP-1 affected glucose uptake by inhibiting Kv1.3, we did

206 detect Kv1.3 mRNA in murine adipose tissue and skeletal muscle (and also liver). Others appear

207   to disagree as to whether Kv1.3 protein is expressed in murine adipose tissue, but this may be

208   because one group used isolated epididymal adipocytes (Li *et al.*, 2006) and the other visceral

209   adipose tissue (Upadhyay *et al.*, 2013). No Kv1.3 protein was detected in murine skeletal muscle

210   (Upadhyay *et al.*, 2013) or in samples of adipose tissue and skeletal muscle from two normal and

211   two type 2 diabetic human subjects (Straub *et al.*, 2011). One possible interpretation of these

212   reports and our findings is that the expression of Kv1.3 is very low in adipose tissue and skeletal

213   muscle, and is sometimes undetectable at the protein level, despite being detectable at the mRNA

214   level. We found more Kv1.3 mRNA in tissues from *ob/ob* than wild-type mice, except for white

215   adipose tissue, but given the relative amounts of adipose tissue in the wild-type and obese

216   animals, the total amount of Kv1.3 mRNA in adipose tissue per animal may be higher in the

217   *ob/ob* mice. We also found more Kv1.5 mRNA in liver and soleus muscle of *ob/ob* compared to

218   lean mice. These results would be consistent with expression of either channel contributing to

219   insulin resistance in *ob/ob* mice.

220   Levels of Kv1.5 mRNA appeared much higher than those of Kv1.3 in both adipocytes and soleus

221   muscle. It is well-established that Kv1.5 is expressed in skeletal muscle (Kang *et al.*, 2009) but

222   its expression in adipose tissue or adipocytes has not been reported. We cannot exclude the

223   possibility that our results are influenced to some extent by the PCR reaction efficiency differing

224   between genes, but this seems unlikely to alter our conclusion that the expression of Kv1.5 was

225   much higher than that of Kv1.3 or Kv1.2 (Irwin *et al.*, 2012). In any event, irrespective of the

226   precise differences in expression of the Kv genes, our findings raise the possibility that Kv1.5

227   was the channel that PAP-1 inhibited to stimulate glucose uptake and inhibit TNFα secretion. If

228   so, Kv1.5 inhibitors might have potential in the treatment of type 2 diabetes. PAP-1 has been

229   reported to inhibit Kv1.5 with an EC_{50} value of 45 nM (Schmitz *et al.*, 2005), but it failed to

230   stimulate glucose uptake in adipocytes when present at a concentration of 300 nM. The 6.7-fold

231   lower EC_{50} value of 45 nM was, however, measured with Kv1.5 from a different species and

232   under very different conditions: it was obtained by applying depolarizing pulses to mouse

233   erythroleukaemia (MEL) cells stably transfected with human Kv1.5 and measuring the reduction

234   in area under the current curve (Grissmer *et al.*, 1994). By contrast, no depolarizing stimulus was

235   applied in our work and we used mouse tissue. Whilst the EC_{50} value of 2 nM for PAP-1 at Kv1.3

236   was also determined by an electrophysiological technique, it did use murine Kv1.3 (Schmitz *et*

237   *al.*, 2005), and it is less likely that the much higher (150-fold) discrepancy between this value and

238   the minimum effective dose of PAP-1 for stimulation of glucose uptake can be explained in terms

239   of the different test systems. PAP-1 is less potent at Kv1.2 (EC_{50}=250 nM) than Kv1.5 (Schmitz

240   *et al.*, 2005), and Kv1.2 mRNA could not be detected in adipocytes using Real Time RCR, ruling

241   it out as a candidate target for PAP-1 in this tissue. We cannot rule out the possibility that Kv1.4,

242   Kv1.6 or Kv1.7 could be the targets of PAP-1 in adipocytes and skeletal muscle, but Kv1.4 is

243   poorly expressed in human adipocytes (You *et al.*, 2013), and 100 nM margatoxin did not

244   stimulate glucose uptake in rat epididymal adipocytes (Beeton *et al.*, 2006), despite having an

245   EC_{50} value for inhibition of Kv1.5 of 5 nM (Garcia-Calvo *et al.*, 1993).

246   We therefore conclude that although Kv1.3 mRNA could be detected in murine white adipose

247   tissue, white adipocytes and red skeletal muscle, inhibition of Kv1.3 does not directly affect

248   glucose uptake in white adipocytes or skeletal muscle. Blockade of Kv1.5 channels in white

249   adipocytes or skeletal muscle might, however, stimulate glucose uptake. Studies using novel

250   Kv1.5 inhibitors might be informative, but their selectivities have been given only relative to

251   other cardiac K channels and not to other Kv1 channels (Ford *et al.*, 2013, Pavri *et al.*, 2012). We

252   do not exclude the possibility that compounds might be useful in the treatment of obesity and

253   insulin resistance, by inhibiting Kv1.3 in other tissues.

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Figure 1

**Effect of PAP-1 on glucose uptake in soleus muscles of male (A) wild-type and (B)
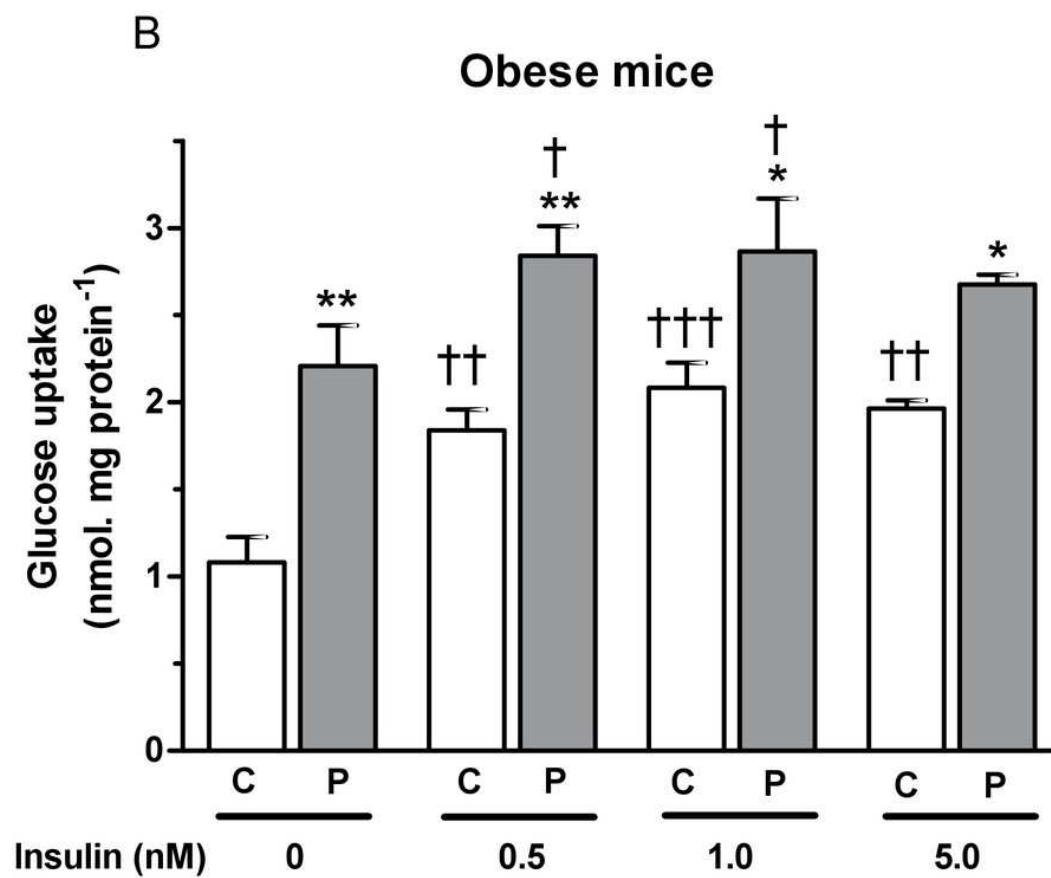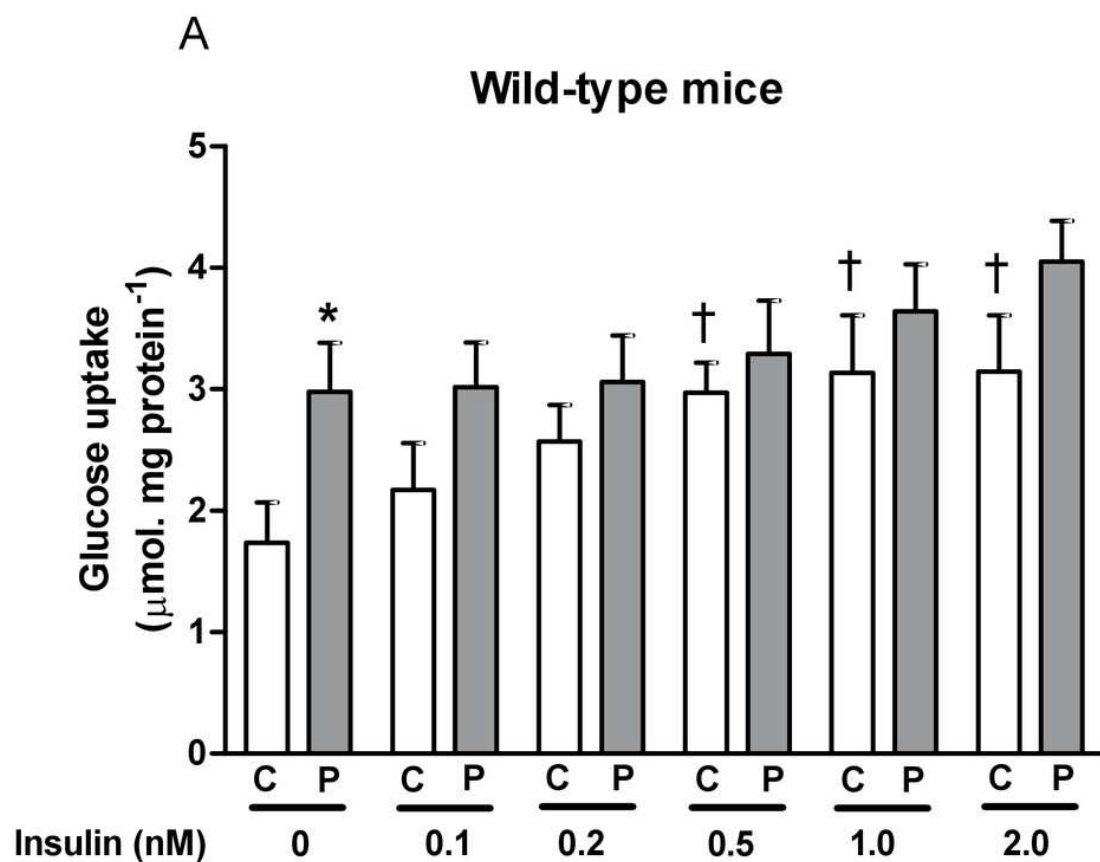*ob/ob* C57Bl6 mice.**

Muscles were treated with insulin (wild-type, 10 nM; *ob/ob*, 100 nM) or PAP-1 (3 microM)
alone, or the combination of insulin and PAP-1. n=8 mice per group for all columns. Data for
each genotype were analyzed by one-way ANOVA followed by Fisher's least significant
difference test. $*P<0.05$ for effect of PAP-1 compared to controls (Con). $^{\dagger}P<0.05$ for effect of
insulin (Ins) compared to controls. $^{\ddagger\ddagger\ddagger}P<0.001$ for the effect of the combination of PAP-1 and
insulin compared to the absence of both compounds (Con). Note that in both wild-type and
ob/ob mice, glucose uptake in the presence of the combination of PAP-1 and insulin was not
significantly greater than in the absence of one of the compounds.

A

Wild-type mice

B

ob/ob mice
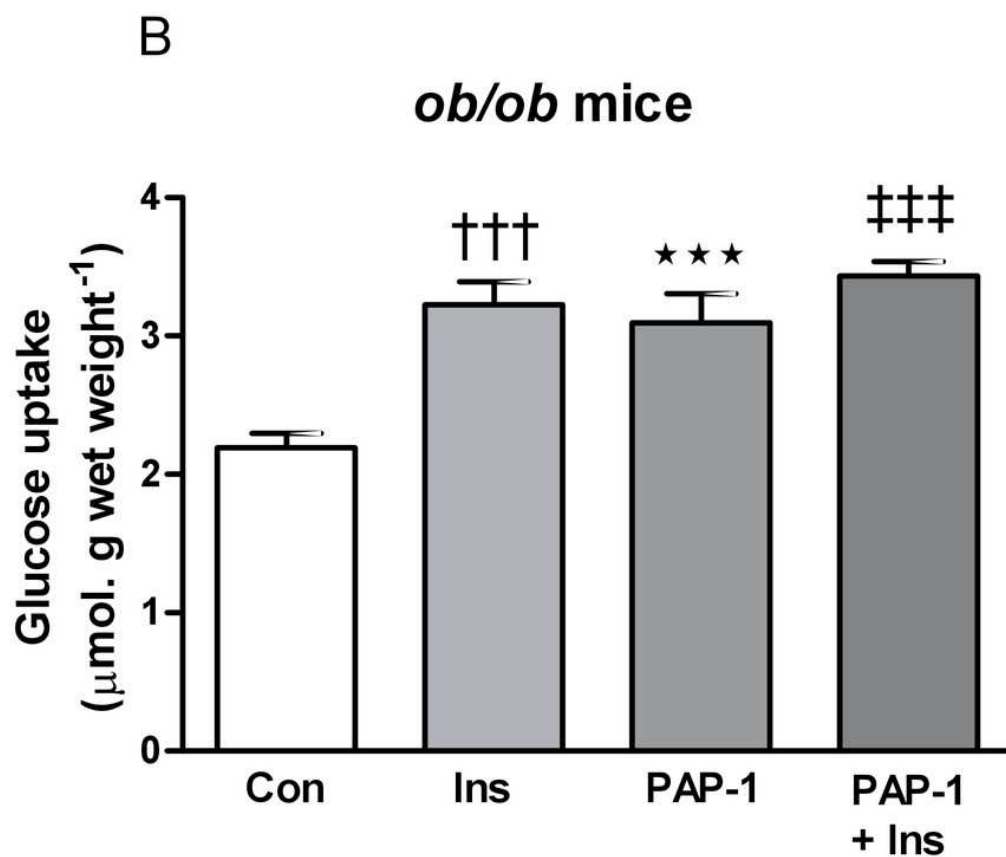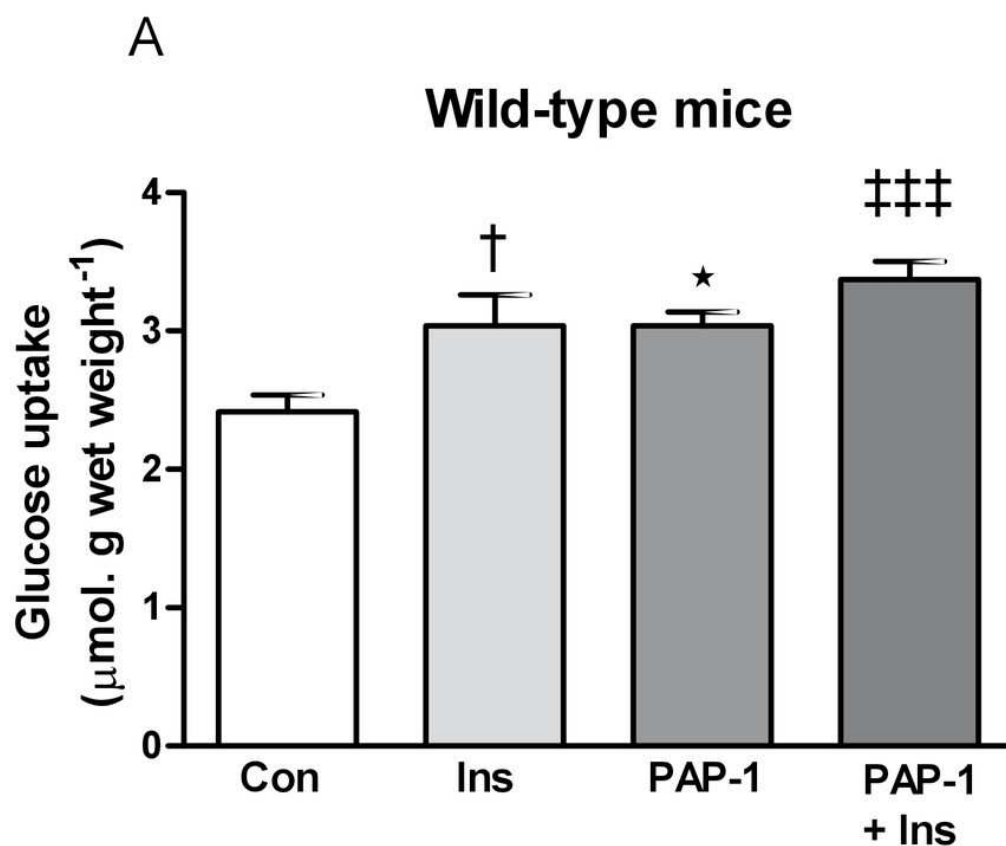
Figure 2

Effect of PAP-1 on glucose uptake in adipocytes of female (A) wild-type and (B) *ob/ob* C57Bl6 mice.

Adipocytes were treated with insulin, PAP-1 (P, 3 microM) alone, or a combination of insulin and PAP-1. n=8 mice per group for all columns. Data for each genotype were analyzed by two-way ANOVA (sources of variation: PAP-1 and insulin concentrations), followed by Fisher's least significant difference test, first for the paired columns at the same concentration of insulin, and second to test for an effect of insulin compared to the value obtained in the absence of insulin but in the presence of the same concentration (0 or 3 microM) of PAP-1. *P<0.05; ***P<0.001 for effect of PAP-1 compared to controls at the same concentration of insulin (C). [†] P <0.05 ; [††] P<0.01; [†††]P<0.001 for effect of insulin compared to no insulin at the same concentration of PAP-1.

A

Wild-type mice

B

Obese mice

Figure 3

Concentration-response curve for the effect of PAP-1 on glucose uptake in the absence of insulin in adipocytes of female wild-type C57Bl6 mice.

n=8 mice per group for all columns. Data were analyzed by one-way ANOVA followed by Fisher's least significant difference test against the control value. *$P<0.05$; *** $P<0.001$ for effect of PAP-1 compared to baseline.
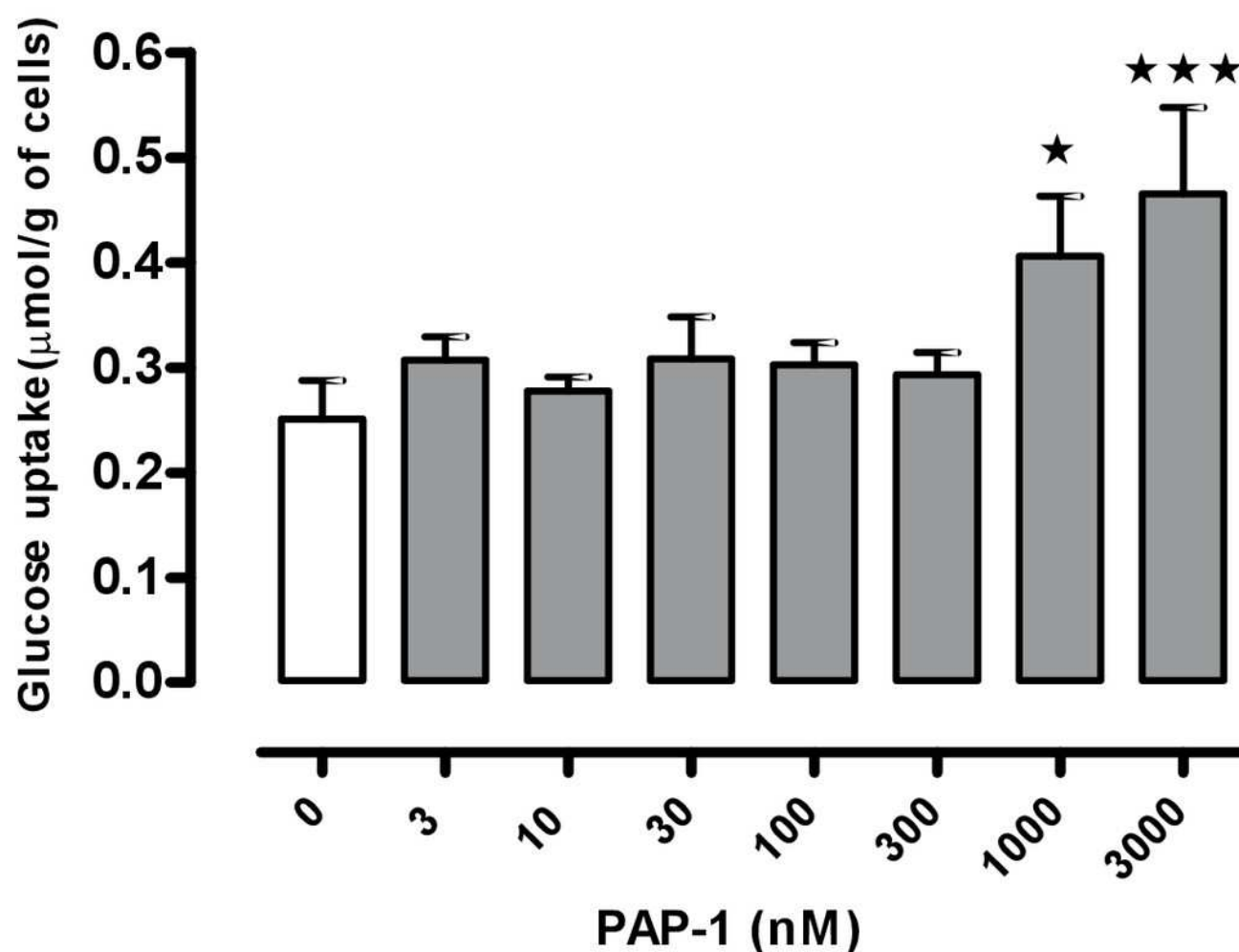
Figure 4

Effect of PAP-1 (3 microM) on TNFα secretion by adipocytes from female wild-type and *ob/ob* mice.

n=6 mice per group for all columns. Data were $\log_{10}$-transformed so that variances were not significantly different between groups prior to analysis by two-way ANOVA (sources of variation: genotype and PAP-1 concentration), followed by Fisher's least significant difference test. *$P<0.05$; **$P<0.01$ for effect of PAP-1 compared to control (Con).
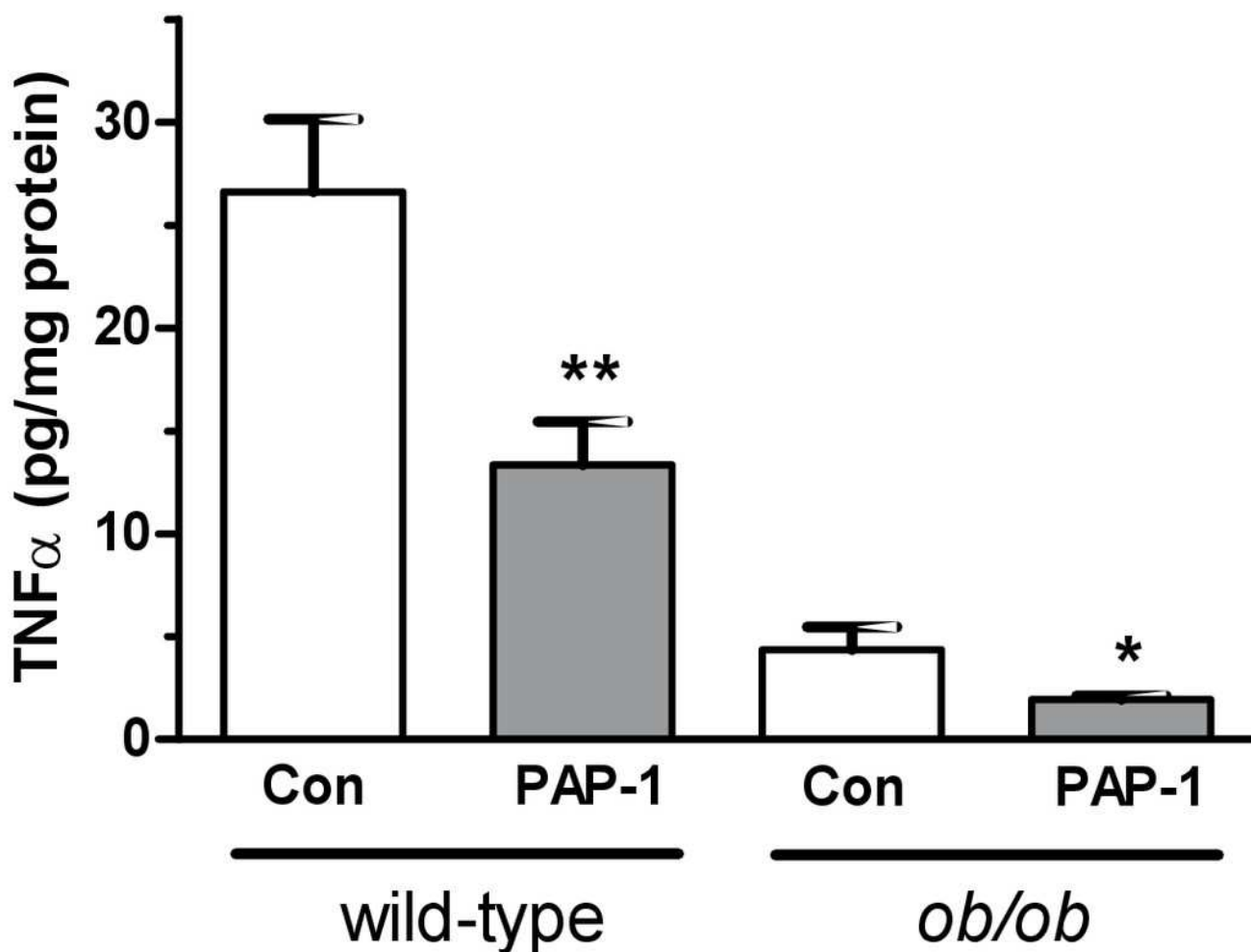
Figure 5

Kv1.3 and Kv1.5 mRNA levels in liver, gastrocnemius, soleus and white adipose tissue of female (A) wild-type and (B) *ob/ob* mice.

n=3 mice for all columns. Expression of each potassium channel was calculated relative to expression of GAPDH in each sample. Data were $\log_{10}$-transformed prior to statistical analysis because the data obtained for Fig 6 suggested that widely distributed data did not follow a normal distribution without this transformation. Analysis was by two-way ANOVA (sources of variation: genotype and tissue) for each gene, followed by Fisher's least significant difference test for each pair of wild-type and *ob/ob* values. Unpaired t-tests conducted on untransformed data gave the same significant differences. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared to wild-type mice.
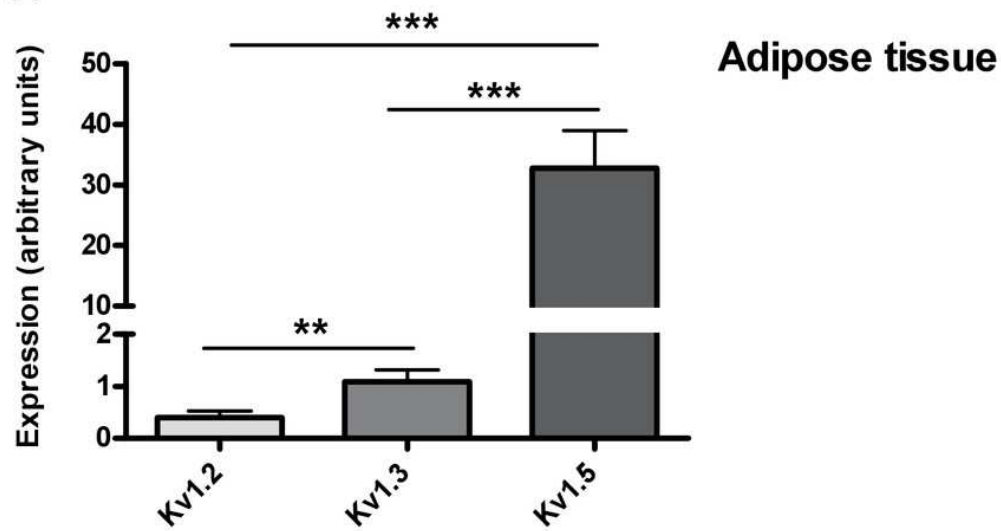
A **Kv1.3**

B **Kv1.5**
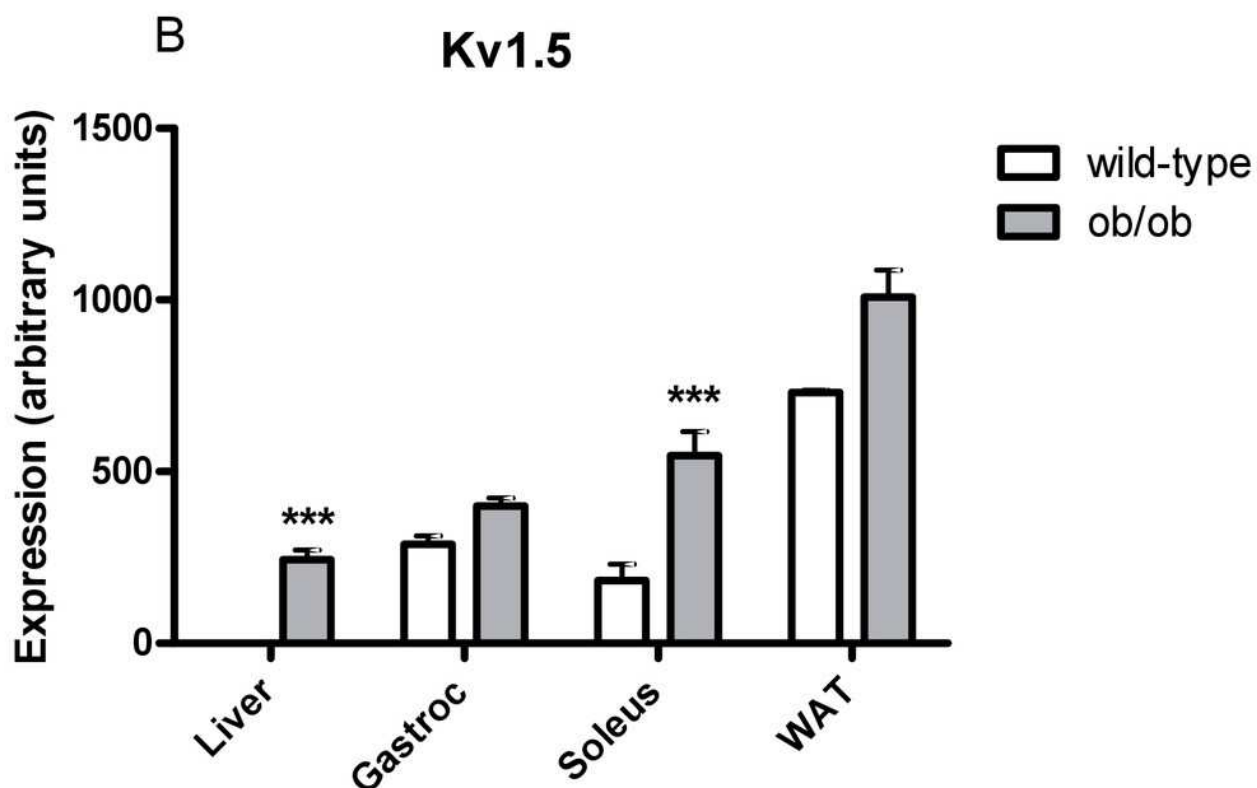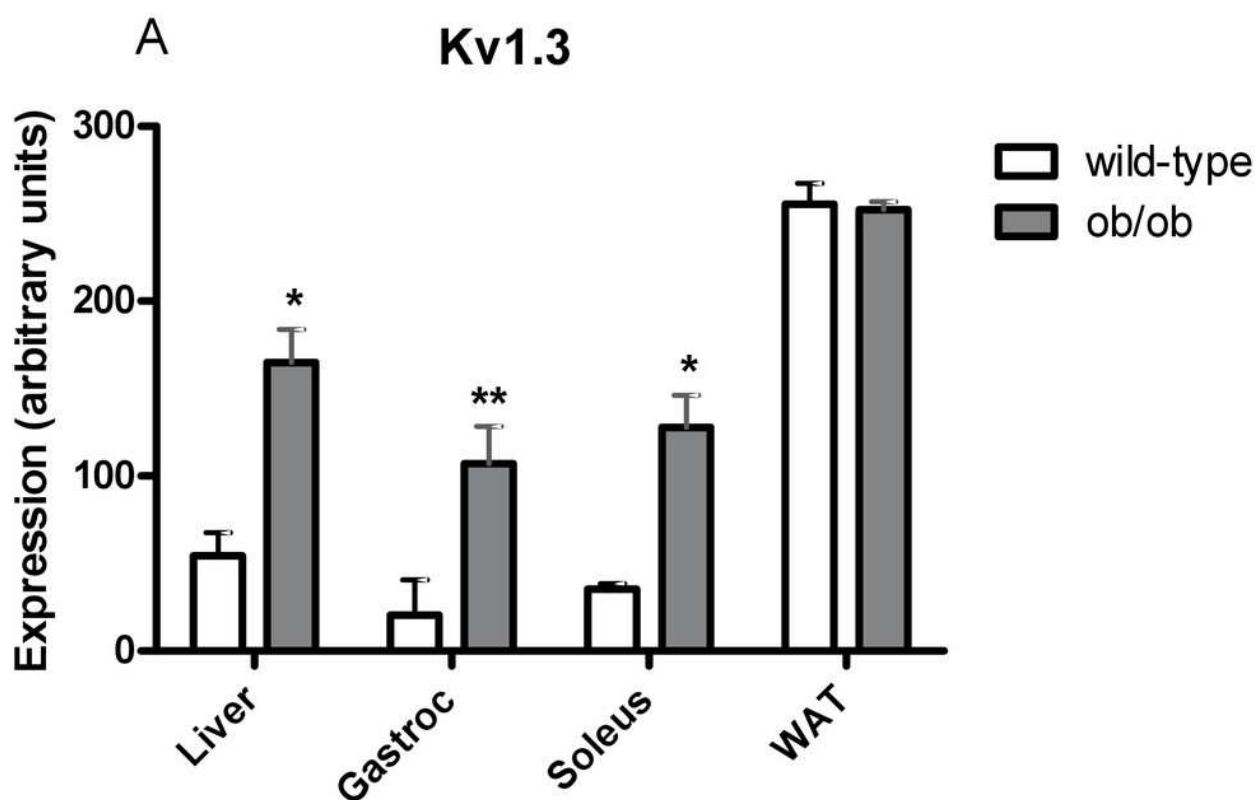
Figure 6

Relative expressionof Kv1.2, Kv1.3 and Kv1.5 expression in (A) adipose tissue, (B) adipocytes and(C) soleus muscle of wild-type mice.

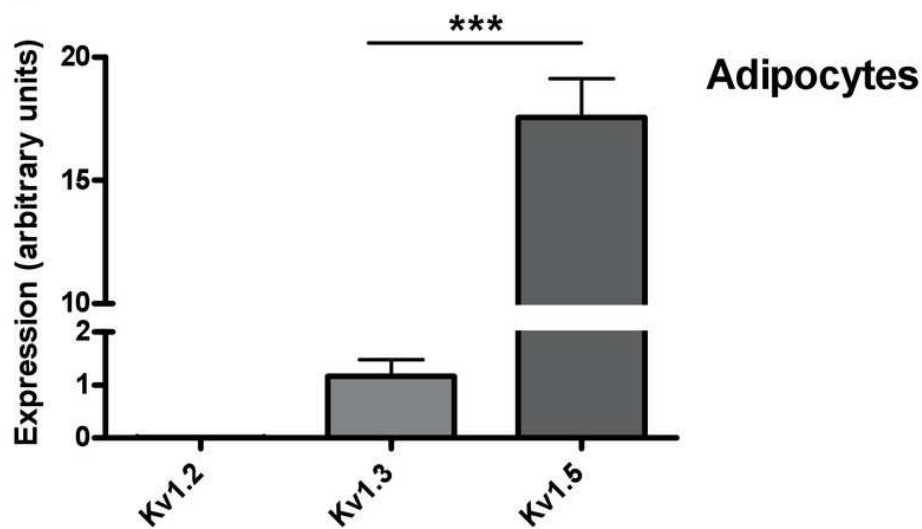Relative expression is given assuming that PCR reaction efficiencies were 1.0 for each gene. The expression of each Kv channel was expressed relative to GAPDH in the same sample and then relative to Kv1.3. ΔC_T GAPDH $- \Delta C_T$ Kv1.3 was 4.43±0.31 for adipose tissue, 6.32±0.34 for adipocytes and 9.8±0.70 for soleus muscle (n = 6). Data were $\log_{10}$-transformed prior to statistical analysis because variances were clearly higher for Kv1.5, which was far more highly expressed than Kv1.2 or Kv1.3. Thus the Bartlett and F-tests showed significant differences in variance for the adipose tissue and adipocyte data respectively when these data were not transformed. Analysis was by two-way ANOVA (sources of variation: gene and tissue), followed by Fisher's least significant difference test for adipose tissue and soleus muscle. Kv1.2 was undetectable in adipocytes, so Kv1.3 and Kv1.5 were compared by unpaired t-test. n=4 mice for soleus muscle and 6 mice for adipose tissue and adipocytes. ** $P < 0.01$; *** $P < 0.001$.

A Adipose tissue

B Adipocytes

C Soleus muscle