Published in final edited form as:

Nat Genet. ; 44(4): 359–360. doi:10.1038/ng.2236.

The role of ATM in response to metformin treatment and activation of AMPK

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To the Editor:

The GoDARTS and UKPDS Diabetes Pharmacogenetics Study Group & The Wellcome Trust Case Control Consortium 2 (WTCCC2) recently reported the first genome-wide association study (GWAS) for treatment response to the anti-diabetic drug metformin in European populations¹. The rs11212617 SNP was found to reach the genome-wide level of significance in the discovery cohort ($n = 1,024$; $P = 1.9 \times 10^{-7}$; odds ratio = 1.64, 95% confidence interval (CI) = 1.37–1.99) and was also shown to be significant ($P < 0.008$) in two replication cohorts (UKPDS and WTCCC2). Among several genes in a 340-kb linkage disequilibrium block containing rs11212617, the authors proposed that ATM, encoding a member of the phosphatidylinositol 3/phosphatidylinositol 4 (PI3/PI4)-kinase family, was the causative gene due to its reported function. In particular, AMP-activated protein kinase (AMPK), the energy sensor widely considered to be involved in the pharmacologic action of metformin, is one of the downstream targets of ATM^{2–4}. The authors showed that an inhibitor of ATM, KU-55933, reduced metformin-stimulated phosphorylation of AMPK in H4IIE rat hepatoma cells, suggesting that ATM has a role in the therapeutic action of metformin.

Our group and others have previously shown (both in vitro and in vivo) that organic cation transporter 1 (OCT1) mediates the entry of metformin into hepatocytes and therefore has an important role in metformin-induced activation of AMPK^{5–7}. Further, we recently showed that tyrosine kinase inhibitors are potent inhibitors of metformin uptake by OCT1, as well as by other organic cation transporters (OCT2, OCT3, MATE1 and MATE2-K)⁸. Examination of the chemical structure of KU-55933, which is also a kinase inhibitor, led us to suspect that it would be a potent inhibitor of OCT1. We hypothesized that the purported action of KU-55933 in reducing metformin-stimulated phosphorylation of AMPK shown in the previous study occurred as a result of inhibition of metformin uptake via OCT1 rather than through a direct effect on metformin-induced activation of AMPK.

We examined the inhibitory effect of KU-55933 (Tocris Bioscience) on [¹⁴C]-metformin (Moravek) uptake in H4IIE rat hepatoma cells (Fig. 1a) and in HEK293 cells stably expressing OCT1 (Fig. 1b). In parallel, we examined the effect of KU-55933 on metformin-induced AMPK activation in H4IIE cells (Supplementary Fig. 1). In the metformin uptake

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AUTHOR CONTRIBUTIONS

S.W.Y. and L.C. performed the experiments and data analysis. S.W.Y., L.C. and K.M.G. conceived the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

assays, we observed that KU-55933 blocked more than 70% of [^{14}C]-metformin uptake in H4IIE cells under the conditions used in the previous study¹ (Fig. 1a). To directly establish that KU-55933 is an inhibitor of OCT1, we also determined its effect on metformin uptake in HEK293 cells stably expressing OCT1. KU-55933 (10 μM) significantly and substantially inhibited [^{14}C]-metformin uptake in OCT1-expressing cells (Fig. 1b). Although results were obtained following 30 min of incubation with KU-55933 and an additional 1 h of exposure to metformin, inhibitory effects of KU-55933 on metformin uptake also occurred at earlier times. However, greater degrees of inhibition were observed at later time points (Supplementary Fig. 2). Consistent with known expression patterns of organic cation transporters in the liver, RT-PCR experiments showed that mRNA levels of rat Slc22a1 (the official symbol for Oct1) were highest in H4IIE cells in comparison to the other organic cation transporters (Slc22a2, Slc22a3, Slc47a1 and Slc47a2) (data not shown). Taken together, these data and the data in Figure 1 suggest that KU-55933 inhibits metformin uptake in H4IIE cells via inhibition of OCT1.

Similar to the authors' findings, we also observed that KU-55933 significantly attenuated metformin-stimulated phosphorylation of AMPK (Supplementary Fig. 1). Known OCT inhibitors from diverse chemical and pharmacologic classes, cimetidine, imatinib and verapamil^{6–8}, reduced metformin uptake in both H4IIE and OCT1-transfected cells (Fig. 1a,b) and, in parallel, reduced metformin-induced AMPK phosphorylation in H4IIE cells (Supplementary Fig. 1a,b). These results are consistent with our previous observations with other OCT inhibitors, verapamil and cimetidine, in mouse and human cells^{6,7}. Given these results, we suggest that KU-55933 attenuates metformin-induced AMPK phosphorylation as a result of its inhibition of metformin uptake into the cells. Our studies in the cell lines suggest that inhibition of OCT1-mediated metformin uptake is the mechanism by which KU-55933 caused inhibition of metformin-stimulated AMPK phosphorylation in H4IIE cells. Of note, at baseline, KU-55933 enhanced the phosphorylation of AMPK in H4IIE cells (Supplementary Fig. 1a,b), perhaps reflecting some nonspecific effect. We noted a similar enhancement in HEK293 cells and mouse fibroblasts exposed to KU-55933 but detected no enhancement in primary hepatocytes from mice (data not shown). It is known that the ATM kinase is activated after the introduction of DNA double-strand breaks⁹; therefore, the activity of ATM is likely to be low in unperturbed cells, such as H4IIE cells.

Another interpretation of the cellular assays described in the previous study, which would be consistent with our studies showing that KU-55933 reduces metformin uptake, would be that KU-55933 inhibited ATM, which in turn reduced OCT1 activity. To determine whether ATM affects the uptake function of OCT1, we transiently transfected *Atm*^{+/+} (A29) and *Atm*^{-/-} (A38) mouse embryonic fibroblasts with a construct encoding mouse Oct1. The results showed that the uptake of metformin was greater in the Oct1-transfected A29 and A38 cells relative to cells transfected with empty vector and that there was no difference in metformin uptake between the two cell lines (Supplementary Fig. 3a). In the presence of KU-55933, Oct1-transfected A29 and A38 cells showed significantly lower metformin uptake compared to untreated cells (Supplementary Fig. 3b,c). These results suggest that the effect of KU-55933 on metformin uptake via Oct1 is independent of *Atm* and that *Atm* does not have a detectable effect on Oct1 activity.

In summary, we were interested to see this first GWAS on metformin response, which reported an association of variants with genome-wide significance at the locus near ATM. Our data suggest that further studies are needed to establish whether ATM or another gene in the ATM locus is the causative gene and, if so, to determine the mechanism by which it modulates the response to metformin. Although metformin has been on the market for many years and is a first-line therapy for type 2 diabetes, the mechanisms of metformin's pharmacologic action, including its target(s), remain controversial^{10–11}. GWAS can suggest

important mechanistic information, and it is critical to perform additional studies to identify and refute or establish causal genes that have a role in the anti-diabetic effects of metformin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge funding from the US National Institutes of Health and the US National Institute of General Medical Sciences (GM61390 and GM36780).

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Figure 1.

Cellular metformin uptake in H4IIE and HEK293 cells. (a,b) The effect of KU-55933 and OCT inhibitors on the uptake of metformin in H4IIE cells (a) and HEK293 cells stably expressing empty vector or human OCT1 (b). Cells were pretreated with DMSO or KU-55933 or other OCT1 inhibitors for 30 min and then treated with various concentrations of metformin (with 10 mM [14 C]-metformin) for 1 h. The amount of [14 C]-metformin in cells was measured. All the compounds except *para*-aminohippuric acid (PAH; a negative control) significantly inhibited metformin uptake (*** P < 0.001, ** P < 0.01, * P < 0.05; unpaired Student's *t* test) compared to cells preincubated with DMSO alone. All data are expressed as mean $\pm$ s.d. from two independent uptake experiments. HEK293 cells stably expressing human OCT1 showed greater OCT1 expression on the plasma membrane compared to cells expressing empty vector⁶.