Many diseases of aging including AD (Alzheimer’s disease) and T2D (Type 2 diabetes) are strongly associated with common risk factors, suggesting that there may be shared aging mechanisms underlying these diseases, with the scope to identify common cellular targets for therapy. In the present study we have examined the insulin-like signalling properties of an experimental AD 8-hydroxyquinoline drug known as CQ (clioquinol). The IIS [insulin/IGF-1 (insulin-like growth factor-1) signalling] kinase Akt/PKB (protein kinase B) inhibits the transcription factor FOXO1a (forkhead box O1a) by phosphorylating it on residues that trigger its exit from the nucleus. In HEK (human embryonic kidney)-293 cells, we found that CQ treatment induces similar responses. A key transcriptional response to IIS is the inhibition of hepatic gluconeogenic gene expression, and, in rat liver cells, CQ represses expression of the key gluconeogenic regulatory enzymes PEPCK (phosphoenolpyruvate carboxykinase) and G6Pase (glucose-6-phosphatase). The effects on FOXO1a and gluconeogenic gene expression require the presence of Zn\(^{2+}\) ions, reminiscent of much earlier studies examining diabetogenic properties of 8-hydroxyquinolines. Comparative investigation of the signalling properties of a panel of these compounds demonstrates that CQ alone exhibits FOXO1a regulation without diabetogenicity. Our results suggest that Zn\(^{2+}\)-dependent regulation of FOXOs and gluconeogenesis may contribute to the therapeutic properties of this drug. Further investigation of this signalling response might illuminate novel pharmacological strategies for the treatment of age-related diseases.

Key words: Alzheimer’s disease, clioquinol, forkhead box O1a (FOXO1a), insulin, Type 2 diabetes, zinc.

INTRODUCTION

Besides its role in glucose homoeostasis, IIS [insulin/IGF-1 (insulin-like growth factor-1) signalling] components are increasingly understood to control complex relationships between diet and longevity in a wide variety of species. In animal models for example, lifelong reductions in IIS increase lifespan [1–3], yet in the clinic, either an enhanced supply of insulin [4] or sensitization to this hormone [5] to alleviate established insulin resistance also improve longevity. In addition, in the last 10 years, results have begun to emerge indicating that many of the risk factors for T2D (Type 2 diabetes), including hyperinsulinaemia and insulin resistance, are also associated with AD (Alzheimer’s disease) (see for example [6]), which has led to growing interest in the possibility of identifying in IIS some common targets for therapy [7,8].

The FOXO (forkhead box O) transcription factor family is recognized as the key downstream effector of IIS longevity signals [2], regulating much of the pleiotropic action of IIS on cell differentiation, proliferation and metabolism [9]. Previous studies established that IIS induction triggers FOXO1a inhibition and exclusion from the nucleus by PI3K (phosphoinositide 3-kinase)-dependent, PKB (protein kinase B)/Akt- and CK1 (casein kinase 1)-mediated phosphorylation of FOXO1a on five residues, Thr\(^{24}\), Ser\(^{256}\), Ser\(^{139}\), Ser\(^{122}\) and Ser\(^{125}\) [10–13]. These residues and nuclear shuttling are conserved in other FOXOs, except for FOXO6, which lacks residues corresponding to Ser\(^{139}\), Ser\(^{122}\) and Ser\(^{125}\) and is predominantly nuclear even when phosphorylated [14]. Our recent studies have investigated Zn\(^{2+}\)-dependent phosphorylation of FOXO1a on these residues in response to tropolone and dithiocarbamate compounds [15], which was accompanied by repression of PEPC (phosphoenolpyruvate carboxykinase) and G6Pase (glucose-6-phosphatase) [15], key regulators of hepatic gluconeogenesis [16–25]. Metal-binding compounds have also been attracting interest as anti-neurodegenerative agents, including the 8-hydroxyquinoline compound CQ (clioquinol). A variety of mechanisms have been proposed to account for the effects of chronic CQ treatment in neurodegenerative disease models, including sequestration of Fe\(^{3+}\)/Fe\(^{2+}\) [26], Cu\(^{2+}\) and Zn\(^{2+}\) [27], Co\(^{2+}\) [28] or alternatively transport of Cu\(^{2+}\) [29] or Zn\(^{2+}\) [30], resulting in cellular effects, including increased levels of metalloproteinase [31], proteasome inhibition [29,32], stimulation of TNF\(_{\text{α}}\) (tumour necrosis factor \(\alpha\)) [33] and regulation of mitochondrial dehydrogenase [28]. Prompted by our interest in the effects of Zn\(^{2+}\)-binding compounds on IIS, in the present study we have investigated the possibility that Zn\(^{2+}\)-dependent regulation of IIS and gluconeogenesis could contribute to the cellular action of CQ.

CQ was used widely in humans until it was withdrawn following cases of subacute myelo-optic neuropathy in Japan [34,35], but the therapeutic prospects for derivatives of this compound might be limited even further because a variety of other 8-hydroxyquinolines were identified in the 1950s as being diabetogenic through the destruction of pancreatic \(\beta\)-cells (Table 1) [36–38]. In common with the effects on IIS
that we report in the present study, the diabetogenic effect of
8-hydroxyquinolines is thought to require interaction with Zn$^{2+}$
[39] but despite this, several Zn$^{2+}$-binding 8-hydroxyquinolines,
including CQ, are non-diabetogenic [36–38,40]. To establish
whether or not Zn$^{2+}$-dependent IIS induction and diabetogenicity
may be dissociated, we have compared the effects on IIS of
a variety of 8-hydroxyquinolines and other structures whose
diabetogenicity is known.

MATERIALS AND METHODS

Materials

8CPT-cAMP [8-(4-chlorophenylthio)-cAMP], aluminium chlor-
ide, 5-amino-8-hydroxyquinoline, 5-chloro-7-ido-8-hydroxy-
quinoline, chromium(III) chloride, copper sulfate, disulfiram, 4-
hydroxyquinoline, 2-hydroxyquinoline, 8-hydroxyquinoline-5-
sulfonic acid, manganese chloride, wortmannin and xanthurenic
acid were all obtained from Sigma–Aldrich. The PKB inhibitor
Akti, PI-103, PD98059 and rapamycin were from Calbiochem.
Zinc acetate was obtained from Riedel de Haen. Dithizone,
8-hydroxyquinoline and quinidine were from Fluka. The com-
ounds used in the present study were dissolved in DMSO
and stored aliquotted (10 μl) at −20 °C. Aliquots were discarded
after one freeze–thaw cycle. All antibodies were as described
previously [12,41], except for anti-pFOXO1a-Ser256, anti-pPKB-
Ser473 (Cell Signaling Technology) and an anti-actin antibody
(Calbiochem/Merck).

Cell culture and lysis

HEK (human embryonic kidney)-293 and HL1c cells were
maintained essentially as described previously [11,41]. HEK-293
cells were used for experiments 4 or 5 days after seeding and
DMEM (Dulbecco’s modified Eagle’s medium)/FBS (fetal bovine
serum) was replaced 1 day before the experiment. HL1c cells were
used for experiments on day 2 after seeding and were serum-starved
on the evening before stimulation. The inhibitors PI-103 and wortmannin were added 1 h before
stimulation, Akti was added 10 min before stimulation and both
PD98059 and rapamycin were added 30 min before stimulation.
Cells were lysed on ice using buffer A [50 mM Tris acetate
(pH 7.5), 1 % (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 10
mM 2-glycerophosphate, 5 mM sodium pyrophosphate, 50
mM NaF, 1 mM sodium orthovanadate, 1 mM benzamidine,
0.2 mM PMSF and 0.1 % 2-mercaptoethanol]. The lysates were
centrifuged at 13 000 g for 5 min at 4 °C, and the supernatants
were removed and stored at −80 °C until use. In all experiments,
cells were serum-starved prior to stimulation for at least 30 min.

RT (reverse transcription)–PCR

RT–PCR assays of insulin-sensitive genes were carried out in
HL1c cells, cultured in DMEM containing 10 % (v/v) FBS and
1g/l glucose. Serum-starved cells were stimulated with agents
for 4 h. RNA was obtained using an RNaseasy® Mini kit from
Qiagen. RNA was reverse-transcribed to produce first-strand
cDNA using SuperScript® II reverse transcriptase (Invitrogen).
Briefly, RNA, dNTPs and random primers were heated at 65
°C for 5 min and then chilled, prior to incubation with the reverse
transcriptase at 25 °C for 10 min and 42 °C for 50 min,
before inactivation by heating at 70 °C for 15 min. RT–PCR
was performed in a 96-well plate with sequence-specific primers
and probes. In each experiment, results were normalized to Dex
(dexamethasone)/cAMP where Dex/cAMP = 100, and, in each
sample, RNA levels were normalized to cyclophilin.

Translational transfection and confocal microscopy

HEK-293 cells were plated in serum-free medium in dishes
containing a sterile glass coverslip. On the following day, cells
were transfected with a GFP (green fluorescent protein)-tagged
wild-type FOXO1a construct which was generated as described
previously [42] using FuGENE 6 Transfection Reagent (Roche).
The transfection mixture was added dropwise to the dish and
incubated overnight. After each treatment, cells were washed with
ice-cold PBS, which was immediately removed and replaced
with ice-cold 4 % (w/v) paraformaldehyde. Cells were fixed in
the dark at room temperature (20 °C) for 15 min, then washed with
ice-cold PBS. Coverslips were mounted on to microscope slides
(VWR) with a small drop of Vectashield (Vector Laboratories)
and fixed into place using nail varnish. Cells were imaged using
a laser scanning confocal imaging system (Leica TCS SP5). The
Leica Application Suite software (Advanced Fluorescence 1.8.2)
was used to capture fluorescent images with a ×63 magnification
oil-immersion lens and ×4 zoom. The nuclei of HEK-293 cells
are large enough to be identified easily without counterstaining.
The intensity of the fluorescence in the nuclear and cytoplasmic
compartments was quantified using the Leica Application Suite
software. A ratio was obtained by dividing the nuclear intensity
value by the averaged cytoplasmic value (two regions of interest
were quantified for the cytoplasm).

RESULTS AND DISCUSSION

CQ and Zn$^{2+}$ induce acute IIS, and phosphorylation of FOXO1a and its exit from the nucleus

Previously, we developed four antibodies that are capable of
detecting endogenous FOXO1a in lysates to study FOXO1a
phosphorylation by PKB/Akt and CK1 in response to insulin
[12,41] and other regulators of the pathway [43,44]. One antibody
detects FOXO1a regardless of phosphorylation state, whereas
the others detect FOXO1a phosphorylated on Thr$^{24}$, Ser$^{256}$
and Ser$^{325}$. PI3K-dependent phosphorylation of Ser$^{325}$
also reported phosphorylation at two priming sites, Ser$^{125}$
and Ser$^{322}$ [12,13]. In the initial experiments, we compared the ability of CQ to induce
IIS in the presence of 10 μM Cu$^{2+}$, Zn$^{2+}$, Cr$^{3+}$ and Al$^{3+}$. We used
HEK-293 cells because phosphorylation of endogenous FOXO1a
is readily detectable in these cells in response to IGF-1 [12] and
other agents [15,44]. Similar to our previous experiments with
disulfiram and tropolones [15], we found that combining CQ with
Zn$^{2+}$ induced IIS after 1 h of treatment, whereas Cu$^{2+}$ was far
less effective and other ions were ineffective (Figure 1A).
In time-course treatments we found that 1 h of treatment induced FOXO
phosphorylation that approached the maximum (Figure 1B),
slower than IGF-1, which induces maximal responses in this
cell line within 5 min [44]. In dose-response experiments, a
concentration of 10 μM approached the maximum (Figure 1C).
Previously, we showed that phosphorylation of FOXO1a triggers
its exit from the nucleus [11–13], and in the present study we found that
CQ/Zn$^{2+}$ induced nuclear exclusion similarly to insulin/IGF-
1 (Figure 2).

Residues Thr$^{24}$, Ser$^{256}$ and Ser$^{319}$ on the FOXOs lie not only
within consensus sequences for phosphorylation by PKB/Akt
and SGK (serum- and glucocorticoid-induced protein kinase),
but also for phosphorylation by p70 S6 kinase and p90RSK
(ribosomal S6 kinase). We found that FOXO1a phosphorylation in response
to CQ/Zn$^{2+}$ was sensitive to two PI3K inhibitors, PI-103 and
wortmannin (Figure 3A). Further experiments in which HEK-
293 cells were incubated with either rapamycin [which prevents
activation of p70 S6 kinase by inhibiting mTOR (mammalian

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### Table 1 Structure of dithizone, 8-hydroxyquinoline and its derivatives

<table>
<thead>
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<th>Compound name</th>
<th>Structure</th>
<th>Diabetogenic in vivo [36–38,40]</th>
<th>Induces phosphorylation of FOXO1a in cells</th>
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<td>+</td>
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<tr>
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<td><img src="image2" alt="Structure" /></td>
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<td>–</td>
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<td>Xanthurenic acid</td>
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<td>n.d.</td>
<td>–</td>
</tr>
</tbody>
</table>
A. R. Cameron and others

Figure 1  Effect of CQ on IIS

(A) Ability of CQ to induce IIS in the presence of a variety of metals. HEK-293 cells were serum-starved for 30 min prior to stimulation with or without 10 μM CQ and the metal ions shown, and then were incubated for 60 min prior to lysis, SDS/PAGE and immunoblotting with the antibodies shown. (B) Time course. Cells were stimulated with 10 μM CQ and 10 μM Zn2+ for the times shown prior to immunoblotting as in (A). (C) Dose-response. Cells were stimulated with various doses of CQ/Zn2+ for 60 min prior to immunoblotting as in (A). Each experiment shown is representative of at least three separate experiments.

Figure 2  Effect of CQ on the cellular localization of GFP-tagged wild-type FOXO1a

HEK-293 cells incubated in serum-free medium (SF) were transfected with GFP-tagged wild-type FOXO1a and after 24 h were stimulated for 60 min with vehicle, 10 μM CQ (Clio), 10 μM Zn2+ (Zn), 10 μM CQ plus 10 μM Zn2+ or IGF-1 (IGF). Cells were then fixed and imaged as described in the Materials and methods section and a nuclear/cytoplasmic ratio was calculated. Quantification is presented in (A), with representative images depicted in (B). Statistical significance was determined at the 95% confidence interval using a one-way ANOVA with respect to the control (*** P < 0.001). Results are means ± S.E.M; n = 20 cells for each condition. The significance of other column-to-column differences is presented above a horizontal line that identifies the two columns.

Effect of quinoline compounds on FOXO1a phosphorylation: critical role of the co-ordinating group at the 8-position

The effect of CQ and Zn2+ led us to investigate the ability of similar compounds to phosphorylate FOXO1a. Many analogues of 8-hydroxyquinoline (Table 1) were identified in the 1950s as being diabetogenic [36–38] and therefore in addition we tested two structurally unrelated diabetogenic substances, alloxan and dithizone. Alloxan is thought not to bind metals, except when transformed to non-diabetic alloxanic acid [47],}

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Regulation of FOXO1a by quinolines

Figure 3  Effect of protein kinase inhibitors on the CQ response

(A) Effect of PI3K inhibitors and (B) inhibitors of mTOR, p90RSK and PKB on the CQ response. HEK-293 cells were placed in serum-free medium with or without pre-treatment with the kinase inhibitors indicated (500 nM PI-103 for 60 min, 200 nM wortmannin for the final 15 min, 100 nM rapamycin for 30 min, 50 μM PD98059 for 30 min, or 10 μM Akti-1/2 for the final 10 min), prior to stimulation with 10 μM CQ and Zn2+ or 100 ng/ml IGF-1, followed by lysis and immunoblotting. Each experiment shown is representative of at least three separate experiments.

but in contrast, the metal-binding properties of dithizone are well known and are thought to contribute to its diabetogenic character [39]. In our screen of these compounds we found that dithizone and two other compounds, 8-hydroxyquinoline and 8-hydroxyquinaldine, strongly induced FOXO1a phosphorylation in a Zn2+-dependent manner (Figure 4A). The availability of analogues of 8-hydroxyquinaldine enabled us to investigate the role of the co-ordinating hydroxy group at the 8-position. In 4-hydroxyquinaldine the co-ordinating group is relocated with respect to 8-hydroxyquinaldine, and in quinaldine it is absent (Table 1). Neither of these compounds was capable of inducing IIS, indicating that the presence of a co-ordinating group at the 8-position is critical for the effect on IIS (Figure 4B). Consistent with this, we found that substitution of the 8-OH group with a non-co-ordinating amino group also generates a compound that is inactive.

Figure 4  Effect of CQ, other hydroxyquinolines and structurally unrelated diabetogenic substances on FOXO1a phosphorylation

(A) Comparison of hydroxyquinolines and structurally unrelated diabetogenic substances on IIS. HEK-293 cells were serum-starved for 30 min prior to treatment with the compounds shown for 30 min. Cells were prepared for immunoblotting with the antibodies indicated as in Figure 1(A). (B and C) Effect of substitution or relocation of the 8-hydroxy co-ordinating group. HEK-293 cells were serum-starved for 30 min prior to treatment with compounds substituted at the 8-position as depicted in Table 1. + C is a positive control for the anti-pSer325 antibody run on the same gel. Lysates were prepared for immunoblotting with the antibodies indicated. Each experiment shown is representative of at least three separate experiments.
Figure 5 Effect of CQ/Zn\(^{2+}\) on gluconeogenic genes compared with a non-metal-binding derivative

(A) Effect of CQ/Zn\(^{2+}\) on gluconeogenic genes. Serum-starved HL1c cells were treated with (ODC) and without (O) 200 nM Dex and 100 μM 8-CPT-cAMP for 4 h. The effects of 10 nM insulin (Ins), 20 μM Zn\(^{2+}\) (Zn), 20 μM CQ or combinations of these agents on Dex/cAMP-induced PEPCK expression (top panel) and G6Pase expression (bottom panel). (B) Dose-response of PEPCK expression (top panel) and G6Pase expression (bottom panel) to CQ treatment. Same as (A), except that cells were stimulated with decreasing doses of CQ plus Zn\(^{2+}\) (μM). (C) Effect of 8-aminoquinoline plus Zn\(^{2+}\) on gluconeogenic genes. Same as (A), except that 10 μM 8-aminoquinoline (8NH\(_2\)) and 10 μM Zn\(^{2+}\) were used. Results are means ± S.E.M. for at least three separate experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA followed by a Tukey post-hoc test. Asterisks indicate significant changes compared with ODC (*** P < 0.001, ** P < 0.01, * P < 0.05). The significance of other column-to-column differences is presented above a horizontal line that identifies the two columns. ns, not significant with respect to ODC. Av, mean.

Effect of CQ on gluconeogenic genes

Next we explored the effect of CQ/Zn\(^{2+}\) and the inactive analogue 8-aminoquinoline on hepatic gluconeogenic gene expression using the cell line HL1c, which we have used previously to measure the expression of these genes [15]. Repression of hepatic gluconeogenesis by reduced expression of PEPCK and G6Pase is recognized as a key aspect of the anti-hyperglycaemic action of insulin [16–25]. Previously, we showed that repression of the gluconeogenic genes PEPCK and G6Pase by the unrelated Zn\(^{2+}\)-binding small molecules disulfiram and β-thujaplicin mirrored the regulation of FOXO transcription factors by these agents [15]. In the present study we found that CQ/Zn\(^{2+}\) also inhibited PEPCK and G6Pase (Figures 5A and 5B). Consistent with the effects on IIS, neither Zn\(^{2+}\) nor CQ was capable of repressing these genes on their own (Figure 5A). In dose-response experiments in the presence of 10 μM Zn\(^{2+}\), CQ inhibited PEPCK and G6Pase with an IC\(_{50}\) of 4 μM (Figure 5B), which is identical with the effect of disulfiram and Zn\(^{2+}\) [15]. Similar experiments carried out with 8-aminoquinoline showed that this compound was ineffective on gluconeogenic genes (Figure 5C).

CQ dissociates FOXO1a regulation from diabetogenic properties

Comparing our results from the present study with previous ones on diabetogenicity [36–38], we found that, among 8-hydroxyquinoline analogues and dithizone, compounds capable of forming uncharged complexes with Zn\(^{2+}\) induce IIS and tended to be diabetogenic, whereas those that do not affect
IIS because they are charged or do not bind Zn$^{2+}$ tended to be non-diabetogenic (Table 1). This suggests that the ability to access the cell and to bind Zn$^{2+}$ is important for diabetogenicity and the ability to induce IIS. It seems unlikely, however, that diabetogenicity is causally linked to IIS-dependent FOXO inhibition because in pancreatic β-cells [48], in common with other tissues such as muscle, IIS induction and FOXO inhibition maintain cell mass and promote cell survival [48,49]. Moreover, in the case of CQ, which protects mice from streptozotocin-induced diabetes [40], IIS induction is dissociated from diabetogenicity (Table 1). Previous studies suggested that 8-hydroxyquinoline acidifies and destroys β-cell insulin-secretory granules by liberating protons following binding of Zn$^{2+}$ [39] but with CQ, acidification may be reduced because the positioning of the electron-withdrawing halogens results in a much lower pK$_a$ value than in 8-hydroxyquinoline. Proton release is not required for Zn$^{2+}$-dependent IIS induction because we have recently found that another Zn$^{2+}$-binding substance DEDTC (diethyldithiocarbamate) [15], which is known to protect against dithizone-induced diabetes [50], and which does not liberate protons on interaction with Zn$^{2+}$ in cells [39], induces IIS at least as potently as dithizone or 8-hydroxyquinoline [15]. Another effect of halogenation is to render CQ more hydrophobic than other 8-hydroxyquinolines, but no more so than dithizone which is also diabetogenic, suggesting that increased hydrophobicity alone is unlikely to account for the lack of diabetogenicity of CQ. Taken together, the results of the present study suggest that there may be scope to design small metal-binding FOXO1a regulators that are non-diabetogenic, particularly by targeting hydrophobic structures that do not liberate protons on interaction with Zn$^{2+}$. It will also be interesting to determine the role of IIS in the opposing effects of streptozotocin and CQ not only in diabetes [48], but also in neurodegeneration, where evidence suggests that intracerebral streptozotocin administration results in pathology resembling AD [51].

Conclusions

In the present study we have investigated the ability of 8-hydroxyquinoline and related compounds to induce IIS and repress gluconeogenic genes. We find that several compounds based on 8-hydroxyquinoline, including CQ, an experimental therapy for AD, are capable of inducing IIS and regulating gluconeogenic genes in a strictly Zn$^{2+}$-dependent manner. This ability to induce IIS does not tolerate absence or relocation of the co-ordinating group at the 8-position, and additional functional groups that change the charge of the molecule also prevented IIS induction, consistent with the notion that the effects are metal-rather than ligand-dependent. Many 8-hydroxyquinolines have previously been reported to be diabetogenic, but our present results with CQ and previously with DEDTC suggest that it is feasible to identify non-diabetogenic Zn$^{2+}$-dependent IIS-inducing agents. CQ has been investigated in experimental models of AD, Parkinson’s disease and Huntington’s disease, and it will be interesting to determine whether or not the IIS responses that we have studied, including FOXO regulation and gluconeogenic gene expression, contribute to the effects of the drug in these disease settings in vivo.

AUTHOR CONTRIBUTION

Amy Cameron carried out most of the blotting and microscopy, and all of the gene expression, experiments. Additional blots were performed by Katherine Wallace, Jean Harthill and Graham Rena. Additional help with microscopy was provided by Lisa Logie, Alan Prescott and Terry Utermann. The paper was written by Graham Rena and then improved in the light of comments from the other authors.

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