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Carboxylesterase converts Amplex red to resorufin: Implications for mitochondrial H₂O₂ release assays

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Original Contribution

1. Introduction

Reactive oxygen species (ROS) are fundamentally involved in aerobic life. They are generated within biological systems and play critical roles in most of them. For instance, there is a wealth of evidence showing that, by causing damage to macromolecules, ROS can contribute to aging processes and to the pathogenesis of multiple diseases [1]. However, they also participate in cell differentiation, tissue regeneration and cellular signalling processes that can activate a multitude of stress responses, which may support survival [review, [2]].

Mitochondria play a central role in cell metabolism and are a major source of ROS in cells [3,4], and are thus involved in many different physiological and pathological processes [5,6]. Therefore, there is a substantial need for simple but reliable and precise techniques to measure mitochondrial ROS production. Probes that change their fluorescence when oxidized provide convenient, sensitive, and versatile means for detecting ROS. Many probes with similar function have been used, such as scopoletin [7], p-hydroxyphenylacetate [8], and homovanillic acid [9]. However, Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine, AR) offers greater sensitivity, lower background and better stability of the resultant fluorescent product, resorufin (7-hydroxy-3H-phenoxazin-3-one) and is thus preferred [10–15] and has been critically evaluated [16]. AR (AH₂) is oxidised to the highly fluorescent resorufin (A) by hydrogen peroxide (H₂O₂) in a 2-electron oxidation reaction catalysed by horseradish peroxidase (HRP), resulting in a 1:1 overall stoichiometry:

\[
\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I}
\]

\[
\text{Compound I} + \text{AH}_2 \rightarrow \text{Compound II} + \text{AH}^* 
\]

\[
\text{Compound II} + \text{AH}_2 \rightarrow \text{HRP} + \text{AH}^* + 2\text{H}_2\text{O}
\]

\[
\text{AH}^* + \text{AH}^* \rightarrow \text{A} + \text{AH}_2
\]
Overall reaction: \( \text{AH}_2 + \text{H}_2\text{O}_2 \rightarrow \text{A} + 2\text{H}_2\text{O} \)

Although \( \text{H}_2\text{O}_2 \)- and HRP-independent oxidation of AR to resorufin has been described (e.g. by nitric oxide and superoxide [17]), this occurs at a considerably lower yield than HRP/\( \text{H}_2\text{O}_2 \)-mediated oxidation. Therefore, in contrast to many other fluorescent dyes directly oxidised by various types of ROS in less specific manners [12], the AR method is generally regarded as allowing full quantification of \( \text{H}_2\text{O}_2 \) from the resorufin fluorescence intensity. Because AR and HRP are widely considered to be incapable of crossing biological membranes [10] (and manufacturers’ information), this method is extensively used to quantify the release of \( \text{H}_2\text{O}_2 \) from mitochondria, and has been instrumental to gaining insights into the mechanism of mitochondrial ROS production [14]. It is also being applied to measure \( \text{H}_2\text{O}_2 \) release from cultured cells and tissue homogenates, as well as in various enzymatic activity assays, as many enzymatic reactions produce \( \text{H}_2\text{O}_2 \).

One caveat of the AR assay that has been experimentally examined is its photosensitivity (reviewed in [18]). However, so far unresolved problems have been noted when the AR method was applied to certain tissues. For example, liver mitochondria result in HRP-independent conversion of AR to resorufin at a high rate even in the absence of respiratory substrate (i.e. with negligible oxygen consumption). This results in the raw quantitative values from liver mitochondria being much higher than those from other tissue mitochondria in similar experimental conditions and with similar oxygen consumption rates. This phenomenon has been discussed in the community but no explanation has been put forward so far. Frequently, it has simply been ignored [15,19–21].

Here we identify carboxylesterase (CES) as an enzyme that converts AR to resorufin without requiring either oxygen, hydrogen peroxide or a peroxidase. We show that contrary to widely held beliefs, mitochondrial membranes are permeable to AR and that AR is converted to resorufin by CES in the matrix of mitochondria from tissues with high CES expression. CES can be inhibited by Phenylmethyl sulfonyl fluoride (PMSF) at doses that do not interfere with either mitochondrial function or the kinetics of the HRP-catalysed oxidation of AR by \( \text{H}_2\text{O}_2 \). Therefore we propose protocols for the quantification of \( \text{H}_2\text{O}_2 \) by the AR method in tissues, cells and mitochondria containing CES. We argue caution in interpreting previous data using the AR methods in such samples. Based on our findings, we speculate that drug metabolism may well be an under-estimated function of mitochondria, especially in tissues such as liver and kidney.

2. Material and methods

2.1. Mice

C57Bl/6 male mice were purchased from Harlan (Blackthorn, UK). C57Bl/6 are a strain of C57Bl/6 kept as a long-established ageing colony at Newcastle [22]. Male mice were housed as described [23]. All work complied with the guiding principles for the care and use of laboratory animals and was licensed by the UK Home Office (PPL60/3864).

2.2. Mitochondria preparation and subfractionation

Mitochondria from liver, brain and skeletal muscle were isolated as described [24]. Liver mitochondria were then purified using percoll gradient [24]. For subfractionation of mitochondria, 1 mg of purified mitochondria were gently mixed with 1 ml of 10 mM Tris/HCL, pH 7.4 to obtain mitoplasts and divided into two aliquots; to one aliquot 2.7 mg proteinase K was added (to shave mitoplasts). Both aliquots were left on ice for 30 min and centrifuged at 12,000g for 10 min at 4 °C. To obtain inner membranes, 100 mM NaCO₃ was added to shaved mitoplasts and left on ice for 30 min, and centrifuged at 100,000g for 15 min at 4 °C. Protein concentration was assessed by BioRad Dc protein assay kit with BSA as standard.

The specificity of the subfractions was confirmed by western blots using Apoptosis inducing factor (AIF) as a marker for intermembrane space, NDUFa9, a subunit of the electron chain transport complex I that is localized in the inner membrane, and glutamate dehydrogenase (GDH), which resides in the mitochondrial matrix.

2.3. AR assay with mitochondria

\( \text{H}_2\text{O}_2 \) release by isolated mitochondria was measured in the assay buffer containing 115 mM KCl, 10 mM KH₂PO₄, 2 mM MgCl₂, 3 mM HEPES, 1 mM EGTA, 0.2% fatty acid free BSA, pH 7.2 at 37 °C, in the presence of exogenous superoxide dismutase (75 U/ml), horseradish peroxidase (HRP) (2 U/ml) and Ampred (50 μM) at 37 °C. The fluorescent intensity of resorufin, the oxidised product of AR, was monitored kinetically in a plate reader (FLUOstar Omega, BMG Labtech) at excitation 544 nm and emission 590 nm. The experiments were protected from light.

For the experiments to test catalase sensitivity to resorufin, the concentration of HRP was lowered to 0.05 U/ml in order for catalase to compete for \( \text{H}_2\text{O}_2 \). Accordingly, the \( \text{H}_2\text{O}_2 \) generating system (i.e. the concentration of mitochondrial) was also lowered so that it did not exceed the capacity of \( \text{H}_2\text{O}_2 \) detection at the given HRP concentration.

Typically, the basal rate (with mitochondria, no substrates) was measured for 8–10 min, then respiratory substrates (either Pyruvate+Malate 5 mM or succinate 4 mM) were added to initiate respiration (and the electron transport chain-linked \( \text{H}_2\text{O}_2 \) release). The fluorescent intensities of the experiments were calibrated against that obtained by the addition of a known amount of \( \text{H}_2\text{O}_2 \) to the experimental media in the presence of AR and HRP.

As inhibitor of HRP-independent conversion of AR to resorufin, 100 μM PMSF (2 μl of 10 mM PMSF in ethanol to the 200 μl total volume) was added to the experimental medium immediately prior to the or during the measurement. Controls received ethanol only.

In the Homovanillic acid (HVA) assay, AR was replaced with HVA (4 mM) while all the other experimental conditions remained identical to the AR assay, and the fluorescent intensity was read at excitation 310 nm and emission 430 nm.

2.4. AR assays with non-mitochondrial samples

To monitor the conversion of AR to resorufin by different tissue samples, various organs were washed to remove blood and homogenized in PBS, and 0.4 mg/ml samples were used in PBS containing 50 μM AR. The conversion of AR to resorufin by Ces1b enzyme was monitored in double distilled water. To create anaerobic conditions, the reaction was carried out in an anaerobic chamber (Belle Technology, UK), with oxygen concentration < 5 ppm.

2.5. Oxygen consumption measurements

Oxygen consumption rates (OCR) by isolated mitochondria were measured in a Seahorse XF24 analyzer (Seahorse Biosciences) as described [24], with either 5 mM pyruvate and malate or 4 mM succinate. The state 3 OCR was achieved by adding 4 mM ADP, state 4 with 2 μM Oligomycin, and uncoupled rates with 4 μM FCCP. 2.5 μM Antimycin was finally added to inhibit...
mitochondrial respiration.

The OCR during conversion of AR to resorufin by Ces1b enzyme was monitored in a high-resolution respirometer, Oxygraph-2k (Oroboros Instruments).

2.6. Chemicals, enzymes and antibodies

AR was purchased from Life Technologies. All other chemicals were from Sigma. Carboxylesterase 1 isoform b human was also from Sigma (E0287); according to the manufacturer, the enzyme was formulated in 0.1 M Potassium phosphate buffer. The antibodies used in western blot experiments were as follows: NDUFA9 (Abcam, ab14713, 1:1000), AIF (Cell Signalling, #4642, 1:1000), GDH (gift from Prof. Robert Lightowlers, Newcastle University, 1:500), CES1 (ab45957, 1:1000).

2.7. Microscopy

3D images of purified mitochondria and mitoplasts stained with AR and mitotracker green (MTG, 100 nm) were acquired using an LSM510 confocal (Zeiss, Germany) and deconvolved using Huygens software (SVI, Netherlands). Subsequently, images were analysed using average line profiles through the central plane of the stack using ImageJ (http://imagej.nih.gov/ij).

2.8. LC-MSMS

20 pmol AR, 20 pmol resorufin standard or 20 pmol of the AR reaction mixture with CES1b in water were analysed at a flow rate of 0.5 ml/min using a gradient from 5% B ((A) 0.1% formic acid in MS grade water, (B) 0.1% formic acid in acetonitrile) to 95% B over 2 min on a Waters Acquity UPLC BFM C18 column (1.7 μM, 2.1 mM x 50 mM). The eluant was monitored using a Waters Xevo G2XS QqTOF mass spectrometer operating at a scan frequency of 10 Hz either in MS mode or in MSMS mode scanning for the precursors 214.0 and 258.0 with a collision energy ramp from 25 to 40 eV. Data were acquired in the range of m/z 50 to m/z 400 at a resolution of 22,000. Using MassLynx 4.1 extracted ion chromatograms were generated for AR (m/z 258.0766), RT (1.13 min) and for resorufin (two peaks were observed at m/z 214.0514 with RTs 0.99 min (unchanged averaged peak area over the whole time course of the experiment) and RT 0.96 min (occurring only after addition of CES1b and increasing over time to saturation)). Scans were averaged across the peaks for each molecule.

2.9. Docking studies

The structures of AR, resorufin and 3,7-dihydroxyphenoxazine were optimized by using the B3LYP functional and the standard 6-31G(d,p) basis set as implemented in Games [25]. Docking simulations involved the resolved CES1 structure in complex with naloxone methiodide, a heroin analogue (PDB id: 1MX9) as well as the already published homology model of CES2 [27]. Although the resolved structure included a homoexameric assembly, the study was focused on only one CES1 monomer, the structure of which was prepared by deleting water molecules and all crystallization additives and then underwent a preliminary optimization maintaining the backbone atoms fixed to preserve the resolved folding. After deleting the bound substrate, the so optimized protein structure was utilized by the following docking simulations by using PLANTS which calculates reliable ligand poses by ant colony optimization algorithm [28]. For both CES isozymes, the search was focused within a 10 Å radius sphere around the key serine residue (Ser221 for CES1 and Ser228 for CES2), 20 poses were generated and scored by ChemPLP function and speed was set equal to 1. The so obtained best complexes were minimized by keeping all atoms outside a 10 Å radius sphere around the bound AR fixed and the optimized complexes were finally used to calculate the parameters required by the predictive equations.

3. Results

3.1. Liver mitochondria convert AR into resorufin requiring neither respiratory chain activity nor HRP

Oxygen consumption by mitochondria at basal rate (state 1, no exogenous substrate added) is minimal and there is very little H$_2$O$_2$ generation. Oxygen consumption and H$_2$O$_2$ generation greatly increase as mitochondria are fuelled with substrate and the electron transport chain activity increases (state 2). Accordingly, the rates of AR oxidation by H$_2$O$_2$ to resorufin were close to zero during state 1 but significantly increased after the addition of substrate, as seen in kinetic measurements from brain or muscle

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**Fig. 1.** Liver mitochondria convert AR to resorufin in the absence of respiration or the catalyst HRP. (a) Traces of a representative experiment measuring conversion of AR to fluorescent resorufin by equal amounts of mitochondria (0.4 mg/ml) from mouse liver (red), muscle (green) or brain (blue) in kinetic mode (time course). Closed symbols indicate the presence of 2 U/ml HRP, while open symbols indicate no HRP in the reaction mixture. Control reactions without mitochondria are denoted by black open circles (no HRP). (b) Average conversion rates of AR to resoru

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mitochondria (Fig. 1a and b). In contrast, in liver mitochondria resorufin was being formed at a high rate even in the absence of respiratory substrate (state 1) (Fig. 1a and b). In brain or muscle mitochondria, resorufin formation required HRP as a catalyst, but in liver mitochondria it occurred in the absence of HRP (Fig. 1a). Furthermore, this reaction was independent of the intactness of mitochondria, because it happened even after a freeze–thaw cycle (Fig. 1c). However, heat denaturation inhibited it (Fig. 1c). The same results were obtained when fluorescence readings were made at end-point instead of employing the kinetic mode, excluding the possibility that artificial photooxidation of AR/resorufin [16,29] was the cause of the HRP-independent oxidation of AR (data now shown).

3.2. PMSF inhibits the HRP-independent conversion of AR

The addition of PMSF, an inhibitor of serine proteases [30], to liver mitochondrial preparations completely inhibited the HRP-independent AR conversion to resorufin (Fig. 2a). This effect was instantaneous. Importantly, the kinetics of resorufin formation from PMSF-treated liver mitochondria showed the typical characteristics of mitochondrial H2O2 release [14,31] as seen for instance in brain mitochondria, namely: (i) resorufin fluorescence did not increase in non-energized mitochondria, (ii) it increased with substrate addition, (iii) blocking the electron flow at complex I by rotenone decreased resorufin fluorescence when a complex II-linked substrate was used, because rotenone inhibited the reverse-electron-flow mediated superoxide production by complex I, and (iv) there was no resorufin fluorescence increase in PMSF-treated mitochondria when HRP was omitted from the reaction (Fig. 2a). Moreover, fluorescence from PMSF-treated liver mitochondria also increased following addition of a complex I-linked substrate. In this case, addition of rotenone enhanced ROS production from complex I, and accordingly the rate of fluorescence increased, albeit weakly (Supplementary Figure S1; see also Discussion). PMSF had no effects on resorufin formation from brain mitochondria under standard experimental conditions where AR was oxidised in an HRP-dependent manner by H2O2 (Fig. 2a). Adding catalase (50 U/ml) to the reaction largely inhibited the oxidation of AR by liver mitochondria in the presence of PMSF and HRP, as in energized brain mitochondria (Fig. 2b). However, the inhibitory effect of catalase was diminished in the absence of PMSF and was completely abolished in the absence of either substrate, HRP or both (Fig. 2b), indicating that the PMSF-inhibitable, HRP-
Fig. 3. PMSF addition enables quantitative measurement of mitochondrial H$_2$O$_2$ release by AR. (a) H$_2$O$_2$ release from purified liver mitochondria measured by the HRP method and PMSF method in parallel experiments. Mitochondria were prepared from 3 young (8 months, ‘Y’) and 3 old (30 months, ‘O’) mice and the experiments were carried out in 4 conditions as follows: pyruvate + malate (PM), pyruvate + malate + rotenone (PM + Rot), succinate (suc) and succinate and rotenone (suc + Rot). The slope of the regression and the intercept are not significantly different from one or zero, respectively. (b) Representative traces from parallel experiments conducted with either AR plus PMSF (AR, left) or Homovanillic acid (HVA, right) on purified liver mitochondria with succinate (4 mM, Suc) as substrate, followed by rotenone (2.5 mM, Rot). Symbols denote the following conditions: liver mitochondria in the presence (diamonds) or absence (circles) of 100 μM PMSF. Ethanol (EtOH) was added as a control; closed symbols indicate presence of HRP in the reaction while open symbols indicate no HRP. (c) Average H$_2$O$_2$ release rates from succinate-energized liver mitochondria with or without rotenone measured with either AR plus PMSF (filled bars) or HVA (open bars). Data are mean ± SD from 3 independent experiments. * denotes $p < 0.05$. (d) Same as (b), but with brain mitochondria. (e) Same as (c), but with brain mitochondria. (f) H$_2$O$_2$ release rates by liver, brain and muscle mitochondria respiring complex I linked substrate (5 mM pyruvate + malate, PM) in the presence of 100 μM PMSF. Representative kinetic traces are shown in state 1 (no substrate), state 2 (after PM) and after blocking the electron flow at complex I by rotenone (2.5 μM, Rot). (g) Quantification of (f). Data are mean ± SD from 3 technical repeats. (h) Same as (f), but with complex II-linked substrate (4 mM succinate, Suc). (i) Quantification of (h). Data are mean ± SD from 3 technical repeats.
independent conversion of AR by liver mitochondria was not
mediated by H₂O₂ release. To assess whether PMSF might impact
on mitochondrial function, we measured oxygen consumption
rates (OCR) of liver mitochondria treated with different con-
centrations of PMSF in states 2, 3, 4 and after complete uncoupling
using FCCP (Fig. 2c and d). Even at a PMSF concentration 5 times
higher than that used for the AR experiments, OCR was unchanged
under all conditions, showing that PMSF did not have adverse
effects on electron transport chain activity. Together, these data
suggest that PMSF inhibited the enzyme(s) responsible for the
HRP-independent conversion of AR to resorufin, while leaving the
capacity for HRP-dependent AR oxidation by H₂O₂ intact.

We realized that HRP-dependent AR oxidation rates (in the
absence of PMSF, calculated as the difference between the rates
with and without HRP) in liver mitochondria should represent AR
oxidation rates measured from PMSF treated mitochondria in the
presence of HRP, that is the AR oxidation specific to mitochondrial
H₂O₂ release: in fact they were very similar (see Fig. 2a). To test
this further, we compared the results from both approaches over a
range of H₂O₂ release rates by liver mitochondria from both young
and old animals, by varying experimental conditions using com-
pound I and II linked substrates, and with and without rotenone.
Over one order of magnitude difference in H₂O₂ release rates, the
results from both methods were in excellent agreement (Fig. 3a).
Moreover, we quantitatively compared H₂O₂ release rates by
PMSF-treated liver mitochondria as measured using AR with an
alternative method, using Homovanillic acid (HVA). HVA is an-
other commonly used HRP-dependent fluorescent probe to
monitor H₂O₂ generation. With this probe, there was no HRP-in-
dependent HVA oxidation in liver mitochondria (Fig. 3b). H₂O₂
release rates with succinate as substrate determined using HVA
were comparable to those of PMSF-treated mitochondria mea-
sured using AR (Fig. 3b and c). Slowing down the rate of H₂O₂
release by addition of rotenone was seen by both methods, but the
rates measured using the HVA method were lower than those
measured using the AR method (Fig. 3c). Brain mitochondria
yielded similar results (Fig. 3d and e), showing a discrepancy be-
tween the two methods when H₂O₂ release rates are low. This
is most probably due to the known lower sensitivity and thus a
higher detection limit of the HVA assay [32]. In agreement with
that, we could not detect H₂O₂ release by brain mitochondria re-
spiring complex I linked substrate with the HVA assay unless ro-
tenone was present, although it was clearly detectable by AR
(Supplementary Figure S2). Taken together, the data indicate that
in the presence of PMSF the AR methods enables for the first time
realistic estimates of H₂O₂ release from mitochondria in different
tissues including liver (Fig. 3f–i).

Frequently, the AR method is used to measure hydrogen per-
oxide release from cultured cells. We measured resorufin fluo-
rescence generated with and without HRP, PMSF and catalase
(Fig. 4a and b) from four cell lines: AML12 mouse hepatocytes;
MRCS, a primary human fibroblast strain; C2C12 undifferentiated
mouse myoblasts; and Hep G2 human hepatocellular carcinoma
cells. All four lines showed significant HRP-independent resoru-
fin production that was completely inhibited by PMSF (Fig. 4a).
HRP-
dependent, PMSF-insensitive resorufin production was sensitive to
catalase (Fig. 4b), indicating that this was a correct indication of
H₂O₂ release. It should be noted that the cell lines rank differently
in terms of PMSF-dependent and -independent resorufin pro-
duction rate in accordance with primary fibroblasts being more
dependent on oxidative phosphorylation (and thus producing
more H₂O₂) than immortalized and tumour cells.

3.3. AR crosses the mitochondrial membrane and is converted to
resorufin in the matrix

To identify the source of the HRP-independent AR conversion
to resorufin by liver mitochondria, we first assessed the possibility
of microsomal contamination. However, our mitochondrial pre-
parations were pure; fewer than 5% of the total of the > 600 de-
dected proteins in purified liver mitochondria were of microsomal
origin [24]. Moreover, confocal imaging of purified liver mito-
ochondrial preparations showed resorufin fluorescence only in
organelles which were also positive for the mitochondrial marker
Mitotracker green (Fig. 5a), thus excluding non-mitochondrial or-
ganelles as sites of HRP-independent resorufin generation. Profile
scanning of the confocal mitochondrial images revealed a wider
signal for Mitotracker Green, while the resorufin fluorescence
was focussed closer to the centre of the mitochondrial images (Fig. 5b),
suggesting an intramitochondrial origin of the resorufin
fluorescence.

In order to determine the submitochondrial localisation of the
site of AR conversion to resorufin, mitoplasts (mitochondria
without the outer membrane), ‘shaved’ mitoplasts (mitoplasts
after removal of intermembrane space proteins), and inner mito-
ochondrial membranes were prepared from purified liver mi-
 tochondria (Fig. 5c). All three fractions were tested for PMSF-
sensitive AR conversion to resorufin. As seen in Fig. 5d, HRP-in-
dependent, PMSF-sensitive AR conversion to resorufin occurred
in intact mitochondria and in shaved and unshaved mitoplasts, but
not in the inner membrane fraction. These observations were
supported by confocal imaging, showing a clear resorufin

![Fig. 4.](https://example.com/fig4)

Fig. 4. Measurement of H₂O₂ release from cultured cells. Cells from the indicated strains were incubated with AR in the absence (a) or presence (b) of HRP and either no
further addition (initial), 100 μM PMSF, or 100 μM PMSF plus 50 U/ml catalase (CAT). Data are mean ± SD from 3 experimental replicates. Asterisks indicate significant
differences (**p < 0.001) between PMSF and initial and between PMSF + CAT and PMSF (cell line-specific ANOVA followed by Holm–Sidak post-hoc test).
fluorescence signal also in mitoplasts (not shown). These experiments showed that the PMSF-sensitive conversion of AR did not occur on the outside of the inner mitochondrial membrane (because the inner membrane fraction was negative) or in the intermembrane space (because rates were equal for mitoplasts and shaved mitoplasts, Fig. 5d). This suggested that AR was able to cross the inner mitochondrial membrane and that the AR-converting activity resided in the matrix. To see whether AR was also able to cross the outer membrane, we incubated liver mitochondria with AR for 10 min without PMSF, allowing for its conversion to resorufin, and divided them into two aliquots. Then the outer membrane from one aliquot of mitochondria was removed and the other served as a control. The resorufin fluorescence intensity of the mitochondria without the outer mitochondrial membrane was identical to the rest of mitochondria (Fig. 5e), confirming that the resorufin signal was not associated with the outer membrane.

To see whether AR can cross mitochondrial membranes in both directions, liver and brain mitochondria were incubated with AR for 10 min at 37 °C, washed twice and then incubated in fresh assay medium with HRP but not AR. External H$_2$O$_2$ (49 pmole) was then added. In this setting, mitochondria are the only source of AR. Only if the washed mitochondria retain AR and release it into the assay medium, can resorufin be generated by HRP-catalysed oxidation by H$_2$O$_2$. This happened for both brain and liver mitochondria (Fig. 5f), albeit the latter displayed a higher basal level of fluorescence and a lower level of resorufin formation following H$_2$O$_2$ addition, suggesting that a significant proportion of AR had already been converted to resorufin in liver mitochondria prior to the HRP-dependent H$_2$O$_2$ assay. This was confirmed if liver mitochondria were pre-treated with PMSF, leading to low basal fluorescence and more pronounced H$_2$O$_2$-induced resorufin formation similar to brain mitochondria (Fig. 5f). Quantification of the resultant resorufin suggested that the amount of retained AR limited H$_2$O$_2$ detection (Fig. 5g). Conversely, when mitochondria were pre-incubated with HRP instead of AR, and washed prior to adding AR (but not HRP) for an H$_2$O$_2$ assay, no conversion of AR to resorufin was observed (data not shown), confirming that AR but not HRP was able to permeate the mitochondrial membrane. Together, these data indicate that AR can transverse the mitochondrial membrane in both directions (either by diffusion or by a transporter-mediated mechanism).

### 3.4. Carboxylesterase catalyses the HRP-independent conversion of AR to resorufin

To identify enzymes that could perform the HRP-independent, PMSF-inhibitable conversion of AR to resorufin, we searched lists of proteins specific to highly purified mouse liver mitochondria [24,33]. This suggested carboxylesterases (CESs) as possible candidates: CESs belong to the serine hydrolase family [34,35], and PMSF targets serine residue in the active site of enzymes [30]. PMSF was reported to inhibit carboxylesterases [36], and other
Michaelis–Menten kinetics [37] which have now been classified as CES. There are 20 isoforms of CES in the mouse with tissue specific distributions [38], participating in the metabolism of xenobiotics, drugs and lipids. Different CES isoforms CES1, CES3, CES5 and CES6 have been found in highly purified mouse liver mitochondria [24,33]. The CES1 gene structure suggests a 50–60% probability for mitochondrial localisation [39]. Different tissues catalyse the HRP-independent conversion of AR to resoru in at different rates, however, all are inhibited by PMSF (Fig. 6a). The tissue distribution of CES1 corresponds well with the PMSF-inhibitable production of resoru (Fig. 6b). Discrepancies in individual tissues (e.g., gut and lung) might be due to cross-reactivities and/or different isoforms being expressed. Within liver mitochondria, CES1 is located in the matrix (Fig. 6c), as is the HRP-independent AR conversion to resoru (see Fig. 5d).

Finally, human Carboxylesterase 1 isoform b (Ces1b) converts AR to resoru in a PMSF-sensitive manner. 100 μM PMSF was added at the indicated time point. (e) PMSF dose response curves for the AR conversion to resoru by Ces1b (Ces1b, black circle) and liver mitochondria (Liver mito, red circle). (f) Michaelis–Menten kinetics showing the rate of resoru production (in pmol/min) by recombinant Ces1b vs AR concentration. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The reaction is inhibited by PMSF with an IC50 similar behaviour to liver mitochondria (Fig. 6e). This reaction resoru-90% of the original AR molecules in the reaction were detected as action product was identical to that of resoru substrate (Fig. 6f). Following its incubation with CES for 28 h, up to 11.0 – 18.0 pmol/min and a K_M of 55 – 12 μM at 12.5 U/ml, indicating that CES behaved like a typical enzyme with AR as a substrate (Fig. 6f). Following its incubation with CES for 28 h, up to 90% of the original AR molecules in the reaction were detected as resoru (Supplementary Figure S3). The UV spectrum of the reaction product was identical to that of resoru (not shown) and the identity of the product was confirmed by LCMSMS (Supplementary Figure S4). The reaction of AR and Ces1b was sensitive to a typical inhibitor for CES1 and CES2, bis(4-nitrophenyl)phosphate (BNPP), resulting in 80–90% inhibition at 1 μM, IC50 ~0.15 μM similar to that of liver mitochondria (Supplementary Figure S5a). Importantly, like PMSF, BNPP at concentrations that inhibited CES efficiently did not impact on mitochondrial respiration (Supplementary Figure S5b), and conversion of AR to resoru in the presence of BNPP required HRP (Supplementary Figure S5c). Loperamide (LPM) is known as a specific inhibitor for CES2, and CES1 has been shown to be insensitive to LPM over a range of concentrations (up to 100 μM) when classic CES substrates were used, such as 4-Nitrophenol acetate, fluorescein diacetate [40] or p-nitrophenyl acetate [41]. However, both the Ces1b- catalysed conversion of AR to resoru in vitro and the conversion catalysed by liver mitochondria showed similar behaviour in the presence of LPM, with only ~50% inhibition of activity observed in the presence of 100 μM LPM (Supplementary Figure S6). These data suggest that CES1, and possibly CES2, can account for AR conversion activity.

To understand the potential reactions between carboxylesterases and AR, we performed docking simulations based on the resolved structure of CES1 in complex with a heroin analogue (PDB Id: 1MX9) and the published homology model of CES2 [27,42]. Simulating the putative complex of AR within the enzymatic cavity of CES1 (Fig. 7a) showed significant complex-
stabilizing interactions, revealing the labile amide moiety of AR in a position conducive to catalysis: Its carbonyl carbon atom approached the hydroxyl group of Ser221, while the carbonyl oxygen atom clearly forms H-bonds with the so-called oxyanion hole that is composed of the backbone atoms of Gly142 and Gly143. The remaining part of the substrate is substantially engaged in hydrophobic contacts. In detail, the phenoxazine ring stabilizes π–π stacking interactions with Phe101, Phe365, Phe426, and His 468, plus hydrophobic contacts involving several apolar side-chains surrounding the AR molecule (e.g. Val254, Leu255, Leu318, Ile359, Ile388 and Met425). Notably, Leu255 and Ala222 also contact the methyl group of the acetyl moiety. In contrast, the two hydroxyl functions were involved in not more than weak H-bonds with backbone atoms (e.g. the 3-hydroxyl approaches the backbone carbonyl group of His468). Together, the molecular docking simulations confirmed the overall stability of the computed complex and revealed the key role played by Van der Waals and hydrophobic interactions in both complex stabilization and undocking processes [43].

Fig. 7b shows the putative complex between AR and CES2. Again, the hydrolysable group of the substrate is placed in a position suitable for catalytic interaction since its carbonyl carbon atom conveniently approaches the key catalytic Ser228 residue. The CES2 subpocket accommodating the phenoxazine ring includes some polar residues and indeed the two hydroxyl functions are engaged in H-bonds with Ser233 and Ser254 while the phenoxazine ring elicits hydrophobic contacts involving Leu151, Leu179, Ile251, and Leu461. The comparison of the two putative complexes reveals some relevant differences mostly focused on the contacts stabilized by the phenoxazine ring. Both enzymes elicit rich networks of hydrophobic contacts which are reinforced by π–π stacking in CES1 and by H-bonds with hydroxyl functions in CES2.

Using these models together with published correlative data [42], pKm values were predicted for the interactions of either CES1 or CES2 with AR (Supplementary Table S1), resulting in excellent agreement with the experimentally measured CES1 pKm value.

The low distances between the labile group of AR and the catalytic serine residues of either CES1 or CES2 (Supplementary Table S1) indicate AR as a possible catalytic substrate for both enzymes. Thus, we propose that CES1 and CES2 are able to convert AR to resorufin in a two-step reaction (Fig. 7c). Enzymatic data and docking simulations suggest that CES1 and CES2 act as amidases, cleaving the amide group of AR, resulting in the release of acetic acid and 3,7-dihydroxyphenoxazine (dihydroresorufin). While we cannot predict how the spontaneous oxidation of 3,7-dihydroxyphenoxazine might occur in physiological conditions, we argue that 3,7-dihydroxyphenoxazine is significantly more reactive than AR or resorufin according to its computed quantum-chemical descriptors (Supplementary Table S2). In the presence of molecular oxygen, 3,7-dihydroxyphenoxazine is readily oxidised to resorufin, forming the basis of the ‘Vanishing Valentine’ classroom experiment [44]. However, the in vitro conversion of AR to resorufin was observed to be as efficient in an anaerobic chamber (oxygen concentration <5 ppm) using degassed water as it was in air (Supplementary Fig. S7a), and the reaction was not inhibited by catalase (Supplementary Fig. S7b). In oxygenated solution in complete darkness, the oxygen consumption detected by high-resolution oxygraphy was very low and not different whether resorufin was produced or not (by blocking the reaction with PMSF) (Supplementary Fig. 7c–e), suggesting that the observed slope of the oxygraph trace was due to electrode drift. In any case, this slope would only equate to less than 0.0002 moles oxygen consumed per mole resorufin formed. While the mechanism of 3,7-dihydroxyphenoxazine oxidation during the CES-catalysed reaction remains unresolved, the oxidation step is unlikely to be rate-limiting.

Fig. 7. CES1 and CES2 can interact with AR. (a) Docking simulation on CES 1 and (b) on CES2 2 for AR. The substrate is shown as balls and sticks with the interacting residues in liquorice. Green colour indicates carbon, blue indicates nitrogen, red stands for oxygen and yellow for sulphur atoms. For clarity reasons, the hydrogen atoms are not displayed. (c) Proposed mechanism for CES to convert AR to resorufin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
4. Discussion

The two main results of our study, namely that mitochondrial membranes are permeable to AR and that carboxylesterases can convert AR to resorufin in a HRP- and oxygen-independent reaction that is inhibited by PMSF, bear a number of interesting consequences. First, they enable the sensitive and quantitative assay of H2O2 release by mitochondria from tissues, cells and mitochondria with high CES content such as liver using the AR method. We showed here that the PMSF-insensitive and the HRP-dependent part of the resorufin fluorescence were quantitative measures of H2O2. PMSF could be substituted by BNPP, as both did not affect OCR, and resorufin formation in the presence of either PMSF or BNPP was fully dependent on HRP, suggesting it was due to HRP-catalysed oxidation by H2O2.

It had previously been claimed that liver mitochondria produced more ROS than mitochondria from other tissues under comparable experimental conditions[15, 19–21] and that liver mitochondria ROS production was insensitive to variations in substrates and ETC inhibitors [45–47]. These are most probably misinterpretations due to the observed high CES activity, which masks the real H2O2-dependent signals. Our results indicated qualitatively and quantitatively similar H2O2 release rates in mitochondria from liver and other tissues (see Fig. 3f–i). Our data revealed for the first time tissue-specific differences in the characteristics of complex I-linked H2O2 release. For example, liver mitochondria are much less sensitive to rotenone in forward-electron flow H2O2 release (i.e. when complex I substrate was used) compared with brain or muscle mitochondria, whereas they are equally sensitive to reverse flow mediated H2O2 release (i.e. ~50% reduction in the rate of H2O2 release when complex II linked substrate was used). We verified that membranes of intact mitochondria are permeable to AR, but not to HRP. Therefore, the notion that the AR method quantitatively detects extramitochondrial release of H2O2 still holds, as long as inhibitors of HRP-independent AR conversion are added to the experiments.

Second, our data provide a rationale for a novel carboxylesterase assay by measuring the PMSF-sensitive and HRP-independent conversion of AR to resorufin. In vitro, CES converts AR quantitatively to resorufin, following strict Michaelis–Mentken kinetics (Fig. 6f). In liver mitochondria ex vivo, this conversion of AR to resorufin changes with the physiological state of the tissue, for instance, it increases with donor age and decreases under dietary restriction (Supplementary Figure S8). However, exactly which isoforms of CES are involved in this reaction in different tissues is unknown.

Finally, our data support the speculation that mitochondria could function as sites of xenobiotic drug metabolism. If AR crosses mitochondrial membranes other molecules may have the same ability. Enzymatic reactions involving CES will frequently produce free radical species, either directly or indirectly. A localisation of carboxylesterases in the mitochondrial matrix with its high concentration of antioxidants might therefore be advantageous for the cell.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2015.11.011.

References
