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Abbreviations: B[a]P: benzo[a]pyrene; GPCR: G-protein coupled receptor; MCT: monocarboxylate transporter; NHE1: Na^+/H^+ exchanger 1; PAH: polycyclic aromatic hydrocarbon; pHe: extracellular pH; pHi: intracellular pH; pHL: lysosomal pH; pHm: mitochondrial matrix pH; ROS: reactive oxygen species; UCP: uncoupling protein

Declarations of interest: none

HIGHLIGHTS

Benzo[a]pyrene, a well-known environmental carcinogen, alters H^+ dynamics.

NHE1 activation by B[a]P plays a key role in H^+ dynamics alterations.

B[a]P-altered pH controls cell death/survival balance via mitochondria and lysosome.

B[a]P impact on H^+ dynamics fits with the malignant tumor-related H^+ gradient reversal.

Altered H^+ dynamics might be universal mechanisms in environmental carcinogenesis.

ABSTRACT

Despite the improvement of diagnostic methods and anticancer therapeutics, the human population is still facing an increasing incidence of several types of cancers. According to the World Health Organization, this growing trend would be partly linked to our environment, with around 20% of cancers stemming from exposure to environmental contaminants, notably chemicals like polycyclic aromatic hydrocarbons (PAHs). PAHs are widespread pollutants in our environment resulting from incomplete combustion or pyrolysis of organic material, and thus produced by both natural and anthropic sources; notably benzo[a]pyrene (B[a]P), i.e. the prototypical molecule of this family, that can be detected in cigarette smoke, diesel exhaust particles, occupational-related fumes, and grilled food. This molecule is a well-recognized carcinogen belonging to group 1 carcinogens. Indeed, it can target the different steps of the carcinogenic process and all cancer hallmarks. Interestingly, H^+ dynamics have been described as key parameters for the occurrence of several, if not all, of these hallmarks. However, information regarding the role of such parameters during environmental carcinogenesis is still very scarce. The present review will thus mainly give an overview of the impact of B[a]P on H^+ dynamics in liver cells, and will show how such alterations might impact different aspects related to the finely-tuned balance between cell death and survival processes, thereby likely favoring environmental carcinogenesis. In total, the main objective of this review is to encourage further research in this poorly explored field of environmental molecular toxicology.

Key words: benzo[a]pyrene; extracellular acidosis; intracellular pH; mitochondria; NHE1; Warburg effect.

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1-INTRODUCTION

Despite the improvement of diagnostic methods and anticancer therapeutics, the human population is still facing an increasing incidence of several types of cancers. According to the World Health Organization, this growing trend would be partly linked to our environment, with around 20% of all cancers stemming from exposure to environmental contaminants, notably chemicals like pesticides, dioxins, polycyclic aromatic hydrocarbons (PAHs) or metals (e.g. arsenic). PAHs are widespread pollutants in our environment resulting from incomplete combustion or pyrolysis of organic material, and thus produced by both natural and anthropic sources; notably benzo[a]pyrene (B[a]P), i.e. the prototypical molecule of this family, that can be detected in cigarette smoke, diesel exhaust particles, occupational-related fumes, and grilled food. This molecule is highly lipophilic and firstly metabolized by the family 1 of cytochromes P450 (CYP1) that are enzymes of the phase I of xenobiotic metabolism. For several xenobiotics, this phase I, along with three other phases (Figure 1), usually carries out the biotransformation of deleterious exogenous molecules to ease their elimination by the organism [1]. However, in some cases, like for B[a]P, the produced metabolites are highly reactive, then forming DNA adducts with a high mutagenic potential. This type of action mechanism (i.e. genotoxicity), along with data from *in vivo* and epidemiological studies, have led the International Agency for Research on Cancer (IARC) to classify B[a]P as group 1 carcinogen for human beings (i.e. *there is enough evidence to conclude that it can cause cancer in humans*) [2]. Actually, numerous studies have shown that B[a]P is a complete carcinogen since it can target all steps of carcinogenesis, that is, initiation, promotion and progression, ultimately leading to cancer and metastasis. Based on literature, it even appears that this PAH can target all the cancer hallmarks defined by Hanahan and Weinberg in 2011 [3], and recently reviewed by our group with respect to B[a]P [4]. Interestingly, H^+ dynamics have been described as key parameters for the occurrence of several, if not all, of these hallmarks [5]. However, data regarding the role of H^+ dynamics during environmental carcinogenesis are still very scarce, and would deserve further research in the future. The present review has thus been undertaken in order to encourage such a research, and will mainly be dedicated to the impact of B[a]P on H^+ homeostasis in liver cells. However, when necessary, data obtained with other environmental carcinogens, especially arsenic, will also be presented.

2-WHAT ARE THE ALTERATIONS IN H^+ HOMEOSTASIS POSSIBLY INDUCED BY ENVIRONMENTAL CONTAMINANTS?

It has long been known that pH_i homeostasis is a key determinant for the maintenance of cellular functions; hence, pH_i has to be tightly controlled. Such a homeostasis relies on both "short-term" (intracellular buffering power) as well as "long-term" regulation systems (transporters of protons or acid equivalents) [6]. Among these latter systems, one can cite the ubiquitous Na^+/H^+ exchanger isoform 1 (NHE1) whose abnormal activation has notably been described as playing a key role in both tumorigenesis and cell death processes [7-11]. This antiporter, located at the plasma membrane, extrudes 1 H^+ ion against 1 Na^+ ion when activated either upon intracellular acidification or upon mitogenic stimulation [12]. This transporter is also known to be sensitive to osmotic changes [13], or modifications in membrane lipid composition [14], and has thus been claimed as a mechano-sensitive transporter [12,13].

2-1-Effects on intracellular pH (pH_i) via NHE1 activation by B[a]P

Using microspectrofluorimetry and the pH-sensitive fluorescent probe carboxy-SNARF-1, we previously found biphasic changes in steady-state pH_i upon exposure of rat hepatic epithelial F258 cells to B[a]P, even at a dose as low as 50 nM. Indeed, a transient intracellular alkalinization was first detected, followed by an intracellular acidification [15]. The use of cariporide, a specific chemical inhibitor of NHE1, allowed us to demonstrate that the alkalinization was due to an early activation of this pH_i regulator by B[a]P, and that NHE1 activation was essential for the secondary acidification to occur [53], through targeting mitochondrial function [16,17]. A B[a]P-induced intracellular alkalinization was also observed in mouse hepatoma cell line Hepa1c1c7 [18]. The next question to be addressed was about the mechanisms underlying NHE1 activation in B[a]P-treated cells. B[a]P, as a lipophilic molecule, has been previously shown to directly increase membrane fluidity since it can very rapidly partition within plasma membrane as soon as cells are exposed to the compound [19]. However, even though such a phenomenon might have occurred under our experimental conditions, it was unlikely to be responsible for the NHE1

activation detected following 48h of exposure to 50 nM of B[a]P; indeed we showed that B[a]P had to be metabolized by CYP1 prior to alter pH_i [15]. Furthermore our work indicated that the increase in membrane fluidity detected upon B[a]P (at 48h with 50 nM) was actually due to NHE1 activation [20]. NHE1 is known to be preferentially located in lipid rafts, that is, membrane nanodomains enriched in cholesterol and sphingolipids [14]. In B[a]P-treated hepatic cells, NHE1 was found to be relocated outside lipid rafts, and this was necessary for NHE1 to be activated, likely through allowing the binding of calmodulin to the C-terminal domain of the transporter [21]; indeed, this binding is known to prevent the action of an autoinhibitory domain in NHE1, thus responsible for activation of the transporter [22,23]. The relocation outside lipid nanodomains was in fact due to a membrane remodeling related to a decreased level of cholesterol; both activation of AhR (aryl hydrocarbon receptor) and production of reactive oxygen species (ROS) resulting from CYP metabolism [15], were involved in this decrease through down-regulation of the expression of 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), a limiting enzyme in cholesterol synthesis [24]. Other mechanisms to explain NHE1 activation upon chemical carcinogen exposure could also be involved, as the case for arsenic trioxide. Indeed, this environmental contaminant, like B[a]P, was shown to activate NHE1 in the Madin-Darby canine kidney (MDCK) cells; in that case, the authors evidenced diverse protein kinases (p42/44 Mitogen Activated Protein Kinases and Protein Kinases Cα, βI and/or μ), as being involved in the exchanger activation [25].

2-2-Effects on extracellular pH (pH_e)

Using the Seahorse technology that measures both oxygen consumption and extracellular pH (pH_e), an extracellular acidification was also detected upon B[a]P exposure, and was found to be concomitant with intracellular alkalinization [26]. Although not tested yet, this decrease in pH_e might have been due either to NHE1 activation or activation of lactate-H^+ co-transport (MCT for monocarboxylate transporter). Indeed, previous studies have reported that both NHE1 and MCT activation, via the transport of H^+ outside cells, can acidify the extracellular microenvironment [27-29]. Regarding the MCT transporter, we previously detected an increase in extracellular lactate concentration from B[a]P-treated hepatic cells [26], which might indicate that this transporter could play a role under our experimental conditions. However, although a role for MCT in the decrease of pH_e has been reported in the short term [30], it might not be the

case in the long term. Indeed, it has been reported that extracellular acidification in tumor cells would be able to inhibit the activity of MCTs; extrusion of lactate could then occur through connexin 43 channels (Cx43; [31]). It is worth noting that we have previously shown that B[a]P can increase gap junction intercellular communication through an impact on the membrane location of Cx43 [32]. A role for NHE1, MCT and Cx43 would thus deserve further investigation regarding the development of extracellular acidification and the increase in lactate efflux upon B[a]P exposure.

2-3-Effects on the pH of organelles

Not only pH_i and pH_e were altered by B[a]P, but so was the pH of organelles. Indeed, based upon data obtained through transfecting a plasmid allowing the mitochondrial expression of a pH-sensitive fluorescent probe, we detected a marked acidification of the mitochondrial matrix with a decrease as large as 1.5 pH unit from a physiological mitochondrial pH (pH_m) ~ 8.0 [26]. Whereas the origin of this acidification remains as yet unknown, one possibility might be *via* activation of the mitochondrial NHE1 (mNHE1). Indeed, mNHE1 activation has been shown to lead to H^+ influx into mitochondrial matrix, notably in cardiomyocytes, which would favor cell survival [33]. Interestingly, this exchanger has been described to be inhibited by cariporide [34]. Another possibility might be *via* an increase in the H^+ leak related to the mitochondrial uncoupling proteins (UCPs), known to be located in the inner membrane. Among these proteins, UCP2 is known as being the most ubiquitous UCP, and appears to be overexpressed in diverse cancers, liver among them [35,36]. This protein can play a role in the control of ROS by lowering the proton-motive force necessary for ATP production by the electron transport chain (ETC; [37]). Interestingly, UCP2 has been shown to be a target of B[a]P; indeed, in neoplastic fibroblasts, this carcinogen appeared to up-regulate the expression of UCP-2 by about 70% [38]. It is noteworthy that UCP2, by inhibiting oxidative phosphorylation, would also favor glycolysis [39], such a metabolic shift (the so-called Warburg effect) being observed upon B[a]P exposure in F258 cells [26].

The pH of lysosomes can also be altered by chemical carcinogens. Indeed, we have shown that in F258 cells, B[a]P could induce an increase in lysosomal pH (pHL) [40]. Such an alkalinization might result from alterations of the physicochemical properties of lysosome

membranes. Indeed, under our experimental conditions, a permeabilization of this membrane was observed, likely resulting from mitochondria-dependent oxidative stress [40]. As a consequence, H^+ ions would leak out of lysosomes. Interestingly, it has recently been reported that increasing lysosomal pH would be able to lead to cytosolic acidification [41]; such a communication between lysosomes and cytosol would involve changes in the interaction between STAT3 (signal transducer and activator of transcription-3) and the vacuolar H^+-ATPase (v-ATPase). In this context, it would be interesting to test whether B[a]P could affect STAT3 expression and localization in our cell model.

In total, it appears that B[a]P can affect pH in different cell compartments (Figure 2). As a consequence, this leads to changes in various pH gradients, notably across plasma membrane, mitochondrial inner membrane and lysosomal membrane. Such changes could have important consequences in terms of cell death and survival, as described in the following section.

3-WHAT MIGHT BE THE CELLULAR CONSEQUENCES OF CARCINOGENS-INDUCED ALTERATIONS IN PH HOMEOSTASIS?

Based upon the important role of pH in the control of various cell processes, notably by acting on enzyme activity and protein-protein interactions, one can easily figure out that any disturbance in pH homeostasis will lead to alterations in cell functions. Chemical carcinogenesis is assumed to result from modifications of the balance between cell death and survival processes, in favor of cells bearing mutations, that are resistant to cell death processes and prone to proliferate. It is worth emphasizing here that a low level of cell death, such as the one induced by a chronic exposure to a low concentration of chemical carcinogens, has been reported as favoring the compensatory proliferation of neoplastic cells [42].

B[a]P possesses the capacity to elicit cell signaling related on one hand to apoptosis, i.e. a specific type of cell death [43], and on the other hand to cell survival [44]. Thus, in F258 cells, we have shown that 50 nM B[a]P could trigger apoptosis, with the involvement of multiple pathways depending or not on the activation of caspases [15,16]. Using cariporide to inhibit

NHE1 activity or silencing RNA to significantly reduce NHE1 mRNA expression, we demonstrated that NHE1 activation, as evidenced by the intracellular alkalinization, played a key role in the apoptosis induced by B[a]P in F258 cells; this was actually in contrast to the largely described role for NHE1 activation in survival processes [5,9,10], and might be explained by the various functions of the transporter (pH regulator, scaffold protein for signalplex, anchor protein for cytoskeleton) [8], as proposed in section 3-2-1. This non-genotoxic pathway is activated in parallel to the classical p53 pathway (i.e. the genotoxic pathway) induced by B[a]P-related DNA damage [15,17]. As described below, both pathways can work together to regulate cell death/survival balance.

In the following sub-sections, we will see how H^+ dynamics and NHE1 can regulate both facets of the cell death/survival balance, through considering different cell compartments as well as microenvironment (Table 1); this could pave the way for a better understanding of environmental carcinogenesis, especially when exposures occur at low doses.

3-1-pH homeostasis and modulation of biophysical characteristics of membranes

Plasma membrane constitutes the first cellular barrier that chemical agents encounter. Our group previously demonstrated that B[a]P can affect membrane fluidity in diverse hepatic cell models [20,45]. Using cariporide, we showed that this membrane fluidization was dependent on NHE1 activation [20]. The underlying mechanisms still remain unknown. NHE1 is directly linked, through its C-terminal domain, to the actin cytoskeleton *via* the ERM proteins (ezrin/radixin/moiesin) [46,47]. Due to this property, one might first propose that NHE1 activation might increase membrane fluidity through remodeling of actin cytoskeleton; indeed, it has previously been shown that cytochalasin B, a molecule known to target actin cytoskeleton, can increase membrane fluidity of polymorphonuclear leukocytes [48]. Another possibility might be through an impact of the alkaline intracellular pH. Indeed, data obtained by Astarie and coworkers [49] indicated that cytosolic pH could vary inversely with the steady-state anisotropy of the fluoroprobe trimethylamino-diphenylhexatriene (TMA-DPH; to measure membrane fluidity near the polar heads of lipids); in other words, an alkalinization would be related to a decrease in anisotropy of the probe, and hence to an increase in fluidity. Such an effect might be due to an effect of low proton concentration towards lipid packing [50]. Finally, we might also

hypothesize that changes in pH on both sides of the plasma membrane might regulate the activity of enzymes involved in lipid synthesis, thereby altering the lipid composition of the membrane and hence fluidity. For example, sphingomyelinases, whose activities depend on pH values, might be activated or inhibited, with consequences in terms of ceramide composition of plasma membrane [51]. Of note, inhibition of acid sphingomyelinase was found to prevent cisplatin-induced membrane fluidization that resulted from an intracellular acidification [52]. In our experimental context, one could thus envision an activation of sphingomyelinases by the B[a]P-induced alterations in H^+ dynamics. Besides changing membrane fluidity, NHE1 activation might also have an impact on the cell surface glycoconjugate composition of cell membrane, with consequences in terms of membrane microstructure. Indeed, due to activation of this transporter, a local decrease of pH_e could occur, thus activating plasma membrane-associated glycohydrolases [53], with consequent alterations in the ceramide content of plasma membrane [54,55]. In this context, it would be worth analyzing the ceramide content of plasma membrane upon B[a]P exposure, and, if any change, its dependence on NHE1.

Regarding the role of the B[a]P-induced membrane fluidization, we have previously shown that prevention of fluidization by membrane stabilizers (eg. cholesterol or ganglioside GM1) significantly inhibited apoptosis [20,56]. Furthermore, we found that the increase in fluidity had consequences on iron homeostasis. Indeed, the increase in iron uptake detected upon B[a]P exposure was markedly inhibited when co-treating cells with cholesterol or exogenous GM1 ganglioside [20,56]. Interestingly, this metal is known to traffic through the endolysosomal pathway [57], and protons have been reported to favor endocytosis, notably by affecting membrane fluidity [58]. It is worth noting that upon B[a]P exposure, alterations of iron homeostasis would play a role not only in the development of oxidative stress, through the Fenton reaction, but also in the activation of the protease activity of lactoferrin, which would then allow caspase activation in F258 cells [40].

3-2-H^+ dynamics and the control of mitochondrial functions

3-2-1-NHE1 and hexokinase II

As NHE1 activation was found to be involved in the B[a]P-induced apoptosis, we then looked for the underlying intracellular mechanisms. Thus, under our experimental conditions, we

found that both NHE1 and p53 worked together to trigger a signaling cascade involving both the protein kinase GSK3α and the oncogene c-Myc, which led to the translocation of hexokinase II (HKII) from mitochondria to cytosol [17,59]. HKII is a key glycolytic enzyme bound to the voltage-dependent anion channel (VDAC) located at the outer mitochondrial membrane [60]. This protein has dual metabolic and apoptotic functions, and its mitochondrial location is determinant for the regulation of these functions [60,61]. Indeed, it favors an energy shuttle between glycolytic ADP and mitochondrial ATP. When HKII interaction with mitochondria is disrupted, electron transport along the ETC is altered and the consecutive electron leakage, combined to oxygen, promotes the production of superoxide anion $O_2^{\cdot-}$ [61]. With respect to B[a]P exposure, we have thus evidenced that enforced HKII mitochondrial expression prevented ROS production as well as apoptosis [17]. The next question still to be addressed is how the couple GSK3α/c-Myc would allow the translocation of HKII from mitochondria to cytosol under B[a]P exposure. Our previous data showed a decrease in c-Myc expression that was involved in HKII translocation induced by B[a]P; furthermore, we found that using a c-Myc-plasmid construct in order to counteract the c-Myc expression decrease, significantly inhibited the B[a]P-induced apoptosis while preventing the reduction of mitochondrial HKII protein level [59]. The implication of c-Myc might have been through its role as a regulator of glucose metabolism, since a reduction of this metabolism has been reported to trigger HKII translocation [62]. However, under our experimental conditions, an increase in glycolysis was rather seen [26], suggesting that other mechanisms might be involved. It has previously been shown that the cholesterol content of mitochondria can regulate the binding of HKII to VDAC, with a loss of this binding as cholesterol content decreases [63]. Besides, c-Myc has been reported to be involved in the regulation of the HMG-CoA reductase expression [64]. As we have shown that B[a]P decreases the expression of this reductase in F258 cells thus leading to a change in cholesterol homeostasis, one might then suppose that the role of the decreased expression of c-Myc in the B[a]P-induced HKII translocation might go through a decrease in the mitochondrial cholesterol content. Finally, note that silencing VDAC1, whose mRNA expression is also down-regulated during lung carcinogenesis in mice treated with B[a]P [65], has been associated with a decrease in c-Myc expression [66]. As a result of the decrease of VDAC expression, one might then expect less HKII bound to mitochondria.

Regarding the role of NHE1 and p53 as regulators of HKII localization and mRNA expression [17,59], the molecular mechanisms involved still remain to be deciphered, especially

as these two proteins were independently activated [17]. It was previously shown by Quach et al. [67] that, in isolated mitochondria, acidic pH increased the release of free HKII from the mitochondrial pellet into the supernatant whereas alkaline pH had the opposite effect. These authors then concluded that a mild alkalinization was sufficient to enhance HK activity by favoring its mitochondrial translocation and VDAC binding, thereby enhancing glycolysis. Taking into account those data, it is then difficult for us to explain the link between NHE1 and HKII *via* the mere increase in pH_i. As NHE1 is also known as capable of forming protein complex for intracellular signaling [47,68,69], one might then suggest the existence of such a complex that would comprise NHE1, GSK3α and p53, all necessary for c-Myc down-regulation [59]. In this context, it would be interesting to analyze the NHE1 interactome under B[a]P exposure.

We have previously shown that inhibiting NHE1 resulted in the prevention of the biphasic change in pH_i, that is, both alkalinization and secondary acidification [15]. As our data suggested a possible involvement of a reversal of the F0F1-ATPase activity (i.e. the ATP synthase or complex V located in mitochondria) in the occurrence of cytosolic acidification upon B[a]P exposure [16], the role of HKII translocation in the reversal of the ATP synthase and acidification would also be worth studying. Regarding the impact of this secondary acidification induced by B[a]P, it was likely involved in the activation of effector caspases and proteases (cathepsins, lactoferrin) as well as in the activation of the endonuclease LEI/L-DNase II, all playing a role in apoptosis [7,16,40].

3-2-2-H^+ dynamics and energy metabolism

Work by Reshkin and coworkers has previously demonstrated a determinant role for the NHE1-dependent intracellular alkalinization in malignant transformation, notably by favoring glycolysis [70]. Alterations in cancer cell metabolism are well recognized as being one of the cancer hallmarks [3], and have been recently reviewed in several papers [71-74]. In this context, cancers are considered as metabolic diseases [75]. Up to now, the metabolic change associated with cancers that has received the most attention, is the so-called Warburg effect [76]. This effect corresponds to a shift of cell metabolism from oxidative phosphorylation (OXPHOS; relying upon mitochondria ETC) to aerobic glycolysis. However, it is clear now that the metabolic reprogramming is more complex than the mere glycolytic shift [73,74,77,78]. Regarding

environmental carcinogens, our previous data indicated that B[a]P, even at the lowest dose of 50 nM, was capable of inducing a pro-survival Warburg effect in several hepatic cell lines; this was detected by an increase in lactate efflux and a decrease in oxygen consumption, the latter reflecting an inhibition of OXPHOS [26]. Interestingly, the alterations of H^+ dynamics reported above appeared to be involved in the B[a]P-induced glycolytic shift. Indeed, in treated F258 cells, an inhibition of the complex II succinate quinone reductase activity (SQR) was observed without any change in succinate dehydrogenase activity (SDH); these effects would lead to a disconnection between the tricarboxylic cycle (also known as Krebs cycle) and OXPHOS [26]. Such changes in complex II activities were previously related to a decrease in pH_m, leading to a dissociation of this complex [79]. As B[a]P exposure also induced an acidification of mitochondrial matrix (from pH_m 8.0 to 6.5 [26]), one might then propose a similar mechanism under our experimental conditions. Another identified target of the acidification of pH_m would be the inhibitory factor 1 (IF1). IF1 is the physiological inhibitor of the F0F1-ATPase; it binds this ATPase when a reverse mode of it is induced upon stress, thereby limiting ATP hydrolysis (see [80] for review). Activation of IF1 has been related to the acidification of pH_m, which favors the dimeric state of the protein, hence allowing the interaction with the β subunit of the pump [81]. We found that B[a]P induced an increase in IF1 content of mitochondria; its silencing not only prevented lactate production, that is, glycolysis, but also increased cell death [82]. Taken all these results into consideration, we can therefore suppose that the change in pH_m we detected might also be involved in the activation of IF1 by B[a]P. Besides a role of pH_m alterations, the NHE1 activation induced by B[a]P also appeared to be involved in the related metabolic reprogramming. Indeed, inhibiting NHE1 by cariporide fully prevented the increase in lactate, that is, glycolysis, observed upon B[a]P exposure [26]. Whereas not studied yet under our experimental conditions, one might suppose a direct involvement of the NHE1-dependent intracellular alkalinization to favor glycolysis. Indeed, it is well known that the activity of the enzymes involved in the glycolytic pathway, eg. phosphofructokinase and lactate dehydrogenase, is sensitive to pH, as reviewed by Reshkin et al. [83]; notably this activity increases while pH_i increases. Besides, the pH gradient between mitochondrial matrix and cytosol would be a key parameter for determining the activity of the transporters involved in OXPHOS; in this context, a reversal of this gradient, as observed with B[a]P (Figure 2), would negatively impact this metabolic pathway [83]. In total, it seems that this is the function of NHE1 as a transporter of H^+ that would be determinant for the metabolic reprogramming induced by

B[a]P, in parallel to changes in pH_m. However, the impact of B[a]P on cell metabolism might also involve regulation of gene expression, as observed upon co-exposing lung epithelial transformed cells to both B[a]P and arsenic [84]. Of note, AhR activation, as triggered upon B[a]P exposure, appears to regulate the expression of several enzymes involved in energy metabolism; this cytosolic receptor is also known to interact with the F0F1ATPase (see [85] for review). In this context, how H^+ dynamics and AhR could act together to induce metabolic reprogramming upon B[a]P exposure remains an open question, as previously emphasized [4].

3-3-B[a]P-induced alterations in autophagy: a role for H^+ dynamics?

3-3-1-Autophagy: a key cellular process to cope with stressful conditions

Macroautophagy, generally referred to as autophagy, is a physiological process, whose main function is to preserve cellular homeostasis by protecting cells exposed to stressful conditions [86,87]. Indeed, this key cellular process will more specifically act by allowing self-digestion of toxic aggregates, damaged structures and organelles, thereby avoiding their toxic accumulation and ultimately allowing their recycling [88,89].

Hence, it is not surprising that any disruption in the fine tuning of this essential quality control process will favor pathogenesis, notably by promoting the accumulation of misfolded signaling proteins and dysfunctional mitochondria [90]. Autophagic dysfunction has thus been related to a wide spectrum of pathophysiological situations, including cancers [91], neurodegenerative [92] and cardiovascular diseases [93]. Considering carcinogenesis, autophagy physiologically acts as a tumor suppressor pathway during the tumor initiation step. However, autophagy has also been shown to sustain cancer progression, metastasis, and resistance to therapies [94,95]. As the present review mainly focuses on H^+ dynamics and environmental carcinogenesis, we will not discuss here the complex and ambivalent contribution of autophagy to cancer progression.

As recently reviewed, a growing number of environmental pollutants are now known to interfere with autophagy [96]. Non-exhaustively, cadmium [97], arsenic [98], paraquat [99], bisphenol A [100], patulin [101] and TCDD [102,103], have been shown to differentially affect autophagy. Of particular interest here, two previous studies have also reported that B[a]P can modulate both autophagy [104] and mitophagy [105].

3-3-2-What impact for B[a]P on autophagy in F258 cells?

Regarding B[a]P and F258 cells, we have shown that exposure of these cells to this PAH induced the conversion of LC3-I into LC3-II involved in the formation and closure of autophagosomes (Figure 3A), after 48 and 36h of exposure with 50 nM and 1 µM of B[a]P, respectively. We also detected a dose- and time-dependent accumulation of the cargo protein p62, whose function is to convey the ubiquitinated proteins in the autophagic vacuoles. For each experiment, chloroquine (CHL), a lysosomotropic compound known to elevate/neutralize the lysosomal/vacuolar pH [106], and to decrease autophago-lysosome fusion [107], was used as a positive control of autophagy inhibition.

The accumulation of autophagic vacuoles was then monitored by fluorescence microscopy, using the Cyto-ID autophagy detection kit (Enzo Life Sciences). As shown in Figure 3B (Green staining), B[a]P (50 nM, 24h) favored such an accumulation. Aggresomes are inclusion bodies that are formed when the ubiquitin–proteasome machinery is overwhelmed, and whose primary function is to confer a cytoprotective balance by sequestering the toxic aggregated proteins, therefore facilitating their degradation by autophagy. We used the PROTEOSTAT Aggresome detection kit (Enzo Life Sciences), based on the detection of ubiquitinated proteins, in order to follow by fluorescence microscopy, the distribution of these structures inside F258 cells exposed to B[a]P (50 nM, 24h). As illustrated in Figure 3B (Red staining), B[a]P-treated cells showed an increase in fluorescence signal, further indicating that B[a]P was responsible for a slowing-down of the aggresome recycling. Taken together, our data suggested that B[a]P exposure of F258 cells might also be responsible for an inhibition of autophagy, leading to an increased accumulation rate of damaged structures (e.g. proteins and organelles).

3-3-3-What role for H^+ dynamics in the B[a]P-elicited autophagic dysfunction?

Although very scarce, the data published so far have indicated that both NHE exchangers and pH_i might be involved in the regulation of autophagy and mitophagy [108,109]. In particular, it has been described that a monensin-mediated alkalinization of pH_i would be able to inhibit mitophagy in SH-SY5Y neuroblastoma cells [109]. As in F258 cells, B[a]P induced an early NHE1-

dependent alkalinization [15], the contribution of NHE1 in B[a]P-elicited autophagic dysfunction was then tested. Using cariporide, we found that inhibiting NHE1 had no effect on the B[a]P-induced dose-dependent accumulation of p62 protein while preventing the conversion of LC3-I to LC3-II (Figure 3C). These results would thus point to a role for the B[a]P-activated NHE1 pathway in the autophagic dysfunction detected in carcinogen-exposed F258 cells. However, the intracellular mechanisms underlying such a role of NHE1 remain to decipher.

Lysosomes are key organelles in the process of autophagy-driven degradation [110]. A first hypothesis considering autophagic dysfunction might therefore be a role for alteration of the lysosomal pH. As a reminder, lysosomal pH increased in B[a]P-treated F258 cells [40]. In addition, in our cell model, lysosomal dysfunction was related to oxidative stress [40], and this latter parameter was notably dependent on NHE1 activation [17]. In this context, it would be interesting to more thoroughly test the role of carcinogen-induced NHE1 activation in alterations of lysosomal pH.

We previously identified that B[a]P could potentiate the expression of IF1, the physiological inhibitor of the F0F1ATPase [82]. As shown by Campanella and coworkers [111], an increase in IF1 in HeLa cells was responsible for an inhibition of autophagy. So a similar mechanism might also occur upon B[a]P, especially as IF1 activation was induced, likely resulting from the decrease in pH_m [26]. Therefore, a role for mNHE1 in the control of autophagy, *via* IF1 activation, would also be worth considering.

B[a]P effects on autophagy might also have their origin in the previously identified effects of this carcinogen on HKII [17]. Indeed, beyond its role in the regulation of glycolysis, HKII is also able to regulate autophagy *via* a direct interaction with mTORC1 [112]. This molecular interaction would allow adjusting the intensity of autophagic flux on the cell metabolic status, with HK2 acting as a switch to integrate both glycolysis and autophagy, thereby increasing cell survival during stressful conditions [112]. As HKII translocation has been found to depend on NHE1 [17], it would be interesting to carry out immunoprecipitation experiments to check if B[a]P is able to slow down autophagic flux by limiting interaction between HKII and mTOR.

Apart from pH, Nitric Oxide (NO) has recently been shown to inhibit autophagy and to promote apoptosis in hepatocellular carcinoma [113]. Thus, NO generation upon B[a]P exposure might be another autophagy-regulating pathway to explore. Indeed, we previously found that

B[a]P exposure resulted in NO production, that was dependent upon the activation of the inducible NO synthase [114].

As recently reviewed, autophagy dysfunction is associated with many of the chemical-induced cytotoxic mechanisms, including mitochondrial dysfunction, DNA damage, oxidative stress, stress of the endoplasmic reticulum, impairment of lysosomal functions, and inflammation [96]. Focusing on B[a]P, our results would thus tend to suggest that activation of NHE1 might play a role in the control of autophagy. Accordingly, one might propose that alterations of H^+ dynamics might be involved in environmental carcinogenesis, notably by limiting the cytoprotective capacity afforded by autophagy, especially in non-neoplastic cells.

3-4-Acidic microenvironment and environmental carcinogenesis: an overlooked link

Even though overlooked regarding B[a]P, the acidification of cell microenvironment, as detected using the Seahorse technology in exposed F258 cells [26], might be involved in different steps of environmental carcinogenesis, such as impairment of DNA repair and of xenobiotic metabolism, inflammation, extracellular matrix degradation and invasion, as well as intercellular communication *via* extracellular membrane nanovesicles. We have previously reviewed several aspects of the role of acidic pH_e in tumorigenesis and cancer therapy [115]; so we will try here to complete the picture, more specifically in link with environmental carcinogens. The readers will also find some more information, notably with respect to the impact of acidic microenvironment on energy metabolism or dormant tumor cells, in more recent reviews [116,117].

3-4-1-Extracellular acidosis and DNA repair

It has previously been shown that a low pH_e can facilitate the acquisition of chromosomal aberrations, i.e. clastogenicity [118]. The maintenance of such aberrations would be facilitated by the fact that a low pH_e could also alter the DNA repair process [119,120]. Interestingly, data obtained by Massonneau and coworkers [121] have recently indicated that even rather small variations of pH_e (from 7.2 to 6.9) could lead to impairment of the DNA repair; as a result, the genotoxicity of double-stranded breaks would be enhanced, leading to genetic instability. In this

context, in cells exposed to an environmental carcinogen like B[a]P, one might expect an increased probability of mutations under acidic conditions, thus favoring the initiation step. Actually, it has been demonstrated by Shi and co-workers [122] that incubating human pulmonary epithelial cells (A549 and BEAS-2B) with nontoxic concentrations of B[a]P at acidic pH_e, resulted in a higher level of B[a]P-DNA adducts and double-stranded breaks. They also reported a negative impact on xenobiotic metabolism, especially on the CYP1 expression and activity. Indeed, they showed that acidic conditions increased the concentration of un-metabolized extracellular B[a]P, and that the level of B[a]P-7,8-diol (i.e. a precursor of reactive metabolites) was significantly higher under acidic *versus* normal physiological conditions [122]. In addition to an effect on the enzymes of xenobiotic metabolism, a low pH_e might also change the capacity of cancer cells to cope with oxidative stress. Indeed, a study by Zhao and coworkers [123] has shown that a long-term exposure (3 months) of colon cancer cells to acidic microenvironment (pH_e 6.5), led to a reduction of ROS level associated with a higher level of reduced glutathione; such an effect was notably related to an up-regulation of glutathione peroxidase.

3-4-2-Extracellular acidosis and vesicle trafficking

Extracellular acidosis might also favor the release of membrane nanovesicles, similarly to what was observed in diverse human cancer cells [124]. These nanovesicles (also referred to as exosomes) are membrane vesicles that are produced by all cells, and whose amount and composition can vary depending on the physiopathological state of cells or the stress conditions [125,126]. These entities play an important role in intercellular communication (notably by carrying lipids, proteins, mRNAs, non-coding RNAs like miRNAs), and are now well recognized as playing a key role in tumorigenesis [127]. Interestingly, recent data show that the low pH_e, a known hallmark of tumor neoplasia, could influence exosome release, thereby allowing the diffusion of malignancy [124]. Moreover, such an increase in exosome release could serve as a means of resistance towards anticancer drugs, as shown for cisplatin; indeed, treated cells would use nanovesicles to extrude this molecule in its native form, thereby limiting its efficacy [128]. Another study also outlined a role for microenvironmental pH in the internalization of exosomes [129]. More specifically, the authors showed that an acidic microenvironment increases the entry of nanovesicles into human melanoma cells. They also demonstrated that the increased

fusion capacity of exosomes released upon extracellular acidification was associated to an increase in membrane rigidity resulting from a change in lipid composition. Under these conditions, it could be useful to target extracellular acidification in order to hamper tumorigenesis as well as tumor resistance carried by exosomes. Interestingly, even though still in its infancy, the study of nanovesicles in the field of environmental carcinogenesis is attracting more and more attention [130,131].

With respect to trafficking of intracellular vesicles, a drop in pH_e has also been described to induce profound changes in lysosome characteristics, with notably a significant shift of lysosomes from the perinuclear region to the cell periphery in various cancer cells [132]. As lysosomes participate in the regulation and function of matrix metalloproteinases (MMPs), serine proteases and cathepsins, which are sequestered in these organelles, such a shift to the cell periphery may facilitate increased secretion of degradative enzymes [132]. Also note that acidic pH is well recognized to participate in the activation of proteases like MMPs and cathepsins, thus favoring the degradation of extracellular matrix [133]. However, the mechanisms linking acidic extracellular pH and lysosome trafficking remain to be determined although a possible role for actin has been put forward. Regarding that point, it is worth noting that certain steps in the actin polymerization process and the actin binding to membrane-anchoring proteins were shown to be associated with changes in pH [134]. With respect to B[a]P, it has been shown that exposure of vascular smooth muscle cells to this carcinogen not only favors the expression of various MMPs, but also migration and invasion [135]. These two latter processes have also been observed with respect to exposure of human hepatocarcinoma cells to B[a]P [136]. Therefore, one might suppose that the B[a]P-induced decrease in pH_e might be involved in the related migration and invasion. In line with this, we previously observed that inhibition of NHE1 prevented the B[a]P-induced migration detected in F258 cells [26]. In this context, it would be worth analyzing the impact of B[a]P on extracellular vesicle production and composition.

3-4-3-Regulation of gene expression

As stated above, it is now well-recognized that an extracellular acidic pH helps the activation of MMPs or other proteases by acting directly on the protein conformation. However,

it has further been shown that pH_e may also control the mRNA expression of several MMP isoforms as well as of cathepsins. Thus, the mRNA expression of MMP-2, -9, -11 and cathepsins B and L, is upregulated under acidic conditions [133,137]. This therefore emphasizes the important role of low pH_e in the digestion and remodeling of extracellular matrix, both being primordial events of the invasive process [138].

Cell exposure to a low pH_e has also been shown to induce the expression of proangiogenic factors such as vascular endothelial growth factor-A (VEGF-A) and interleukin-8 (IL-8) [133,139], thus indicating that the acidic tumor microenvironment also contributes to tumor angiogenesis and progression. Another function sensitive to extracellular pH, with potential important consequences on tumor progression, is the inflammatory response; indeed, an extracellular acidification can modulate both pro- and anti-inflammatory responses, notably by acting on the expression of diverse cytokines [140-142]. In addition, the low microenvironmental pH_e inside tumors represents a key mechanism in immune escape and in the obstruction of immune mechanisms to attack the tumor [143]. However, regarding inflammation, gene transcription would not be the only target for acidic pH_e [144]. In line with this, more recent studies have found that acidic extracellular environment could also affect the miRNA expression, both *in vitro* and *in vivo* [145,146]. Circulating miRNAs, like Let-7a that regulates several genes linked to inflammation [147], might also be affected by extracellular acidosis [148]. Therefore, it seems that an acidic pH_e can impact the expression of miRNAs not only within cancer cells but also outside (i.e. in biological fluids), with possible consequences at a distant site of the initial tumor.

The next question that arises is how an extracellular acidification can impact gene and miRNA expressions. Despite the evidence for the involvement of different transcription factors [115], only a few studies have considered how extracellular H^+ can activate them, and more precisely how cells can sense the acidic pH to trigger a signaling cascade. Besides a possible role for H^+-sensitive ion channels, notably resulting in calcium signaling [149], it has been shown that several G protein-coupled receptors (GPCR) could also be involved as pH_e sensors. This is the case for example of the ovarian cancer G-protein-coupled receptor 1 [OGR1] and the G2A that respond to extracellular acidification [150,151]. The activation of such GPCRs by acidic pH_e might involve a change of their trafficking within cells, with more receptors present in the plasma membrane, as reported for OGR1 in leukocytes [152]. The result of such an activation might

then be an accumulation of cyclic AMP to control proinflammatory cytokine production [140,153] or the triggering of calcium signals [140,154]. Other H^+-sensitive GPCRs like GPR4 have also been shown to be implicated in intestinal inflammation in a mouse model of acute experimental colitis [155]. With respect to cancers, it is worth stressing that not only tumor cells exhibit a high expression of such receptors, as for example in human medulloblastoma tissues [154], but also potent carcinogenic molecules may affect their expression. This is the case for example of B[a]P that has been found to increase the expression of GPR68 in exposed macrophages [156]. In this context, one might suppose that the B[a]P-exposed cells would then be more sensitive to an acidic microenvironment than normal counterparts, due to the increase in H^+-sensing GPCR expression. This point would be worth exploring in the future. Of note, GRP68 has been recently proposed as an emerging drug target in cancer [157].

4-CONCLUSION

Our works on B[a]P have clearly evidenced changes in H^+ dynamics upon exposure to low doses of this carcinogen (i.e. doses more relevant to human exposure), likely relying, for most of them, on activation of NHE1. This activation that occurs in parallel to the canonical genotoxic pathway, has been found to have important consequences in terms of regulation of the balance between cell death and survival, notably by acting on mitochondria and energetic metabolism. Thus, the impact of B[a]P on H^+ dynamics is in line with the proton gradient reversal (high pH_i/low pH_e) known to be a selective and new hallmark of malignant tumors. Apart from B[a]P and arsenic salts, it can then be suggested that the carcinogenic activity of many other carcinogens of different origins and natures could share the same and/or similar pathways and effects on cellular H^+ dynamics, thus leading to the possibility for NHE1 and/or other proton extruders' upregulation and overexpression to be universal mechanisms in environmental carcinogenesis. Based upon the fact that alterations in H^+ dynamics are related to several, if not all, cancer hallmarks, these studies therefore emphasize the need to further consider this important parameter when analyzing the carcinogenesis process induced by environmental pollutants. Indeed, such investigations could lead to the identification of new targets in order to prevent, or at least limit, deleterious impacts of these xenobiotics.

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FIGURE LEGENDS

Figure 1. **The different phases of xenobiotic metabolism.** Following cell exposure, a xenobiotic (X) will be transformed into a molecule that will be easily eliminated. This transformation occurs through several phases that notably involved different enzymatic activities such as cytochrome P450s (CYPs) and glutathione-S-transferases regarding phases 1 and 2, respectively. However such a multi-enzymatic process can also lead to the production of reactive metabolites (X*) and reactive oxygen species (ROS), which then can lead to deleterious effects towards several cell constituents.

Figure 2. **Impact of benzo[a]pyrene (B[a]P) on H^+ dynamics**. Upon B[a]P exposure, the H^+ dynamics have been shown to be altered, thus resulting in variations in intracellular (pH_i) and extracellular pH (pH_e) as well as in the pH of both mitochondrial matrix (pH_m) and lysosomes (pH_L). All these changes lead to either disappearance or reversal of pH gradient across membranes (as illustrated by dotted arrows), when compared to physiological conditions.

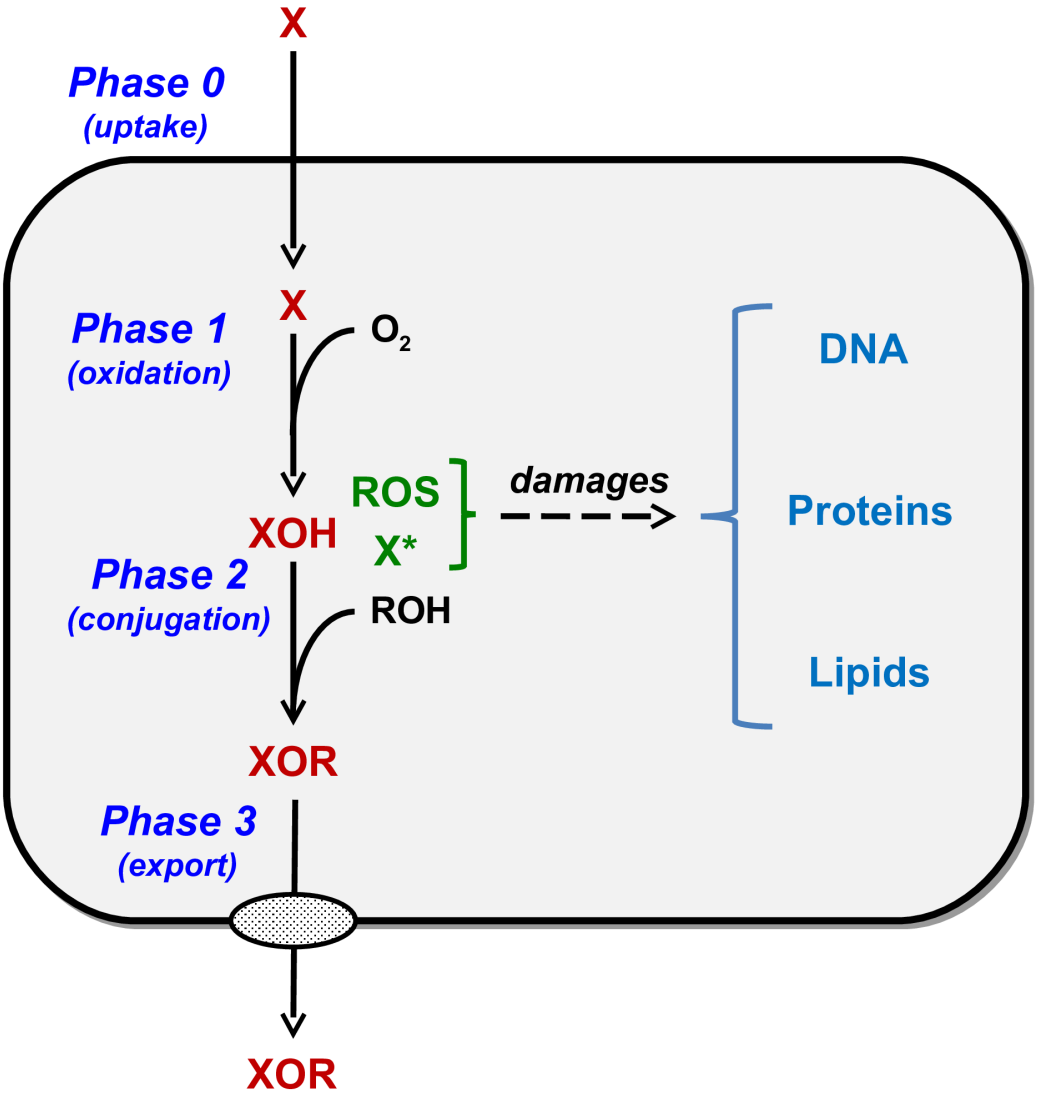
Figure 3. **Impact of B[a]P exposure on the autophagy process in rat hepatic epithelial F258 cells.** Cells were treated or not with 50 nM or 1 µM B[a]P for 36-72h ***(A)***, 24h ***(B)*** or 48h ***(C)***. Western-blots show the expression of p62 and LC3-II, two autophagic markers, in presence ***(C)*** or not ***(A)*** of cariporide, the specific inhibitor of NHE1. Autophagic vacuoles and aggresomes were visualized by fluorescence imaging in cells treated either with B[a]P (50 nM, 24h) ***(B)***. Chloroquine (CHL, 10 µM) was used as positive control of autophagy.

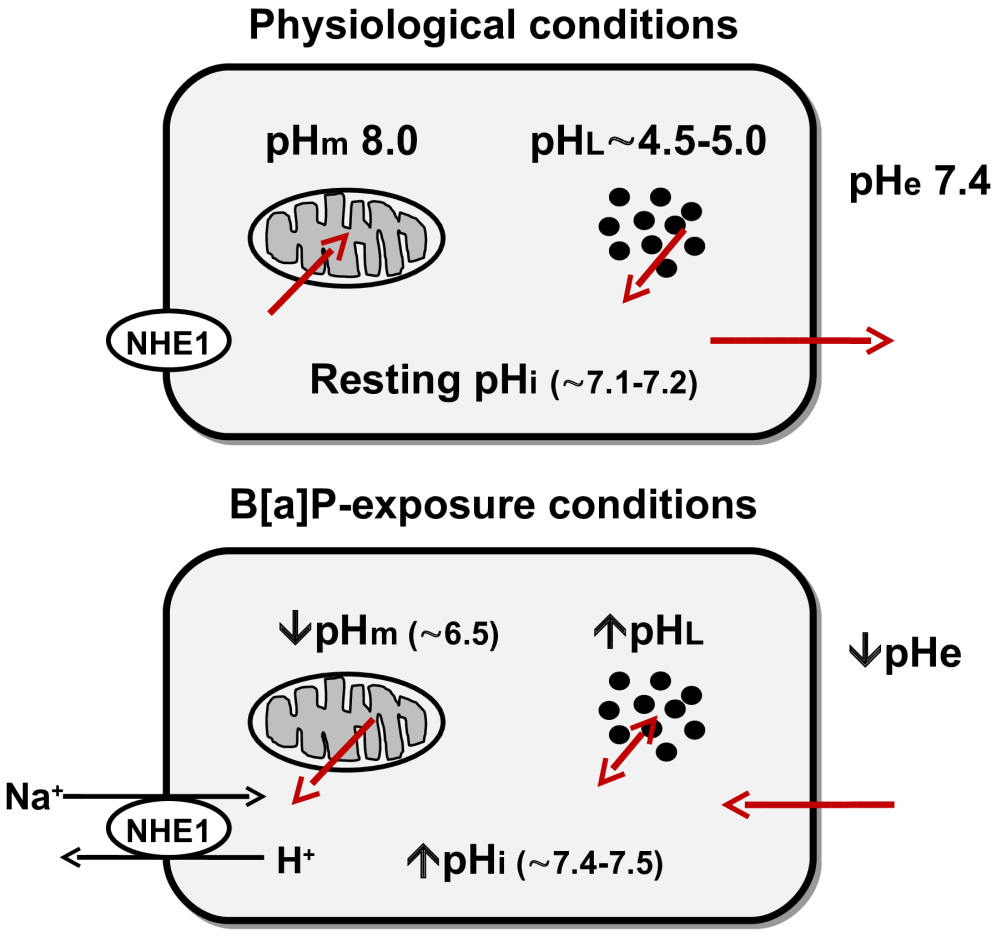
Table 1: Possible targets for alterations in H^+ dynamics due to environmental carcinogens.

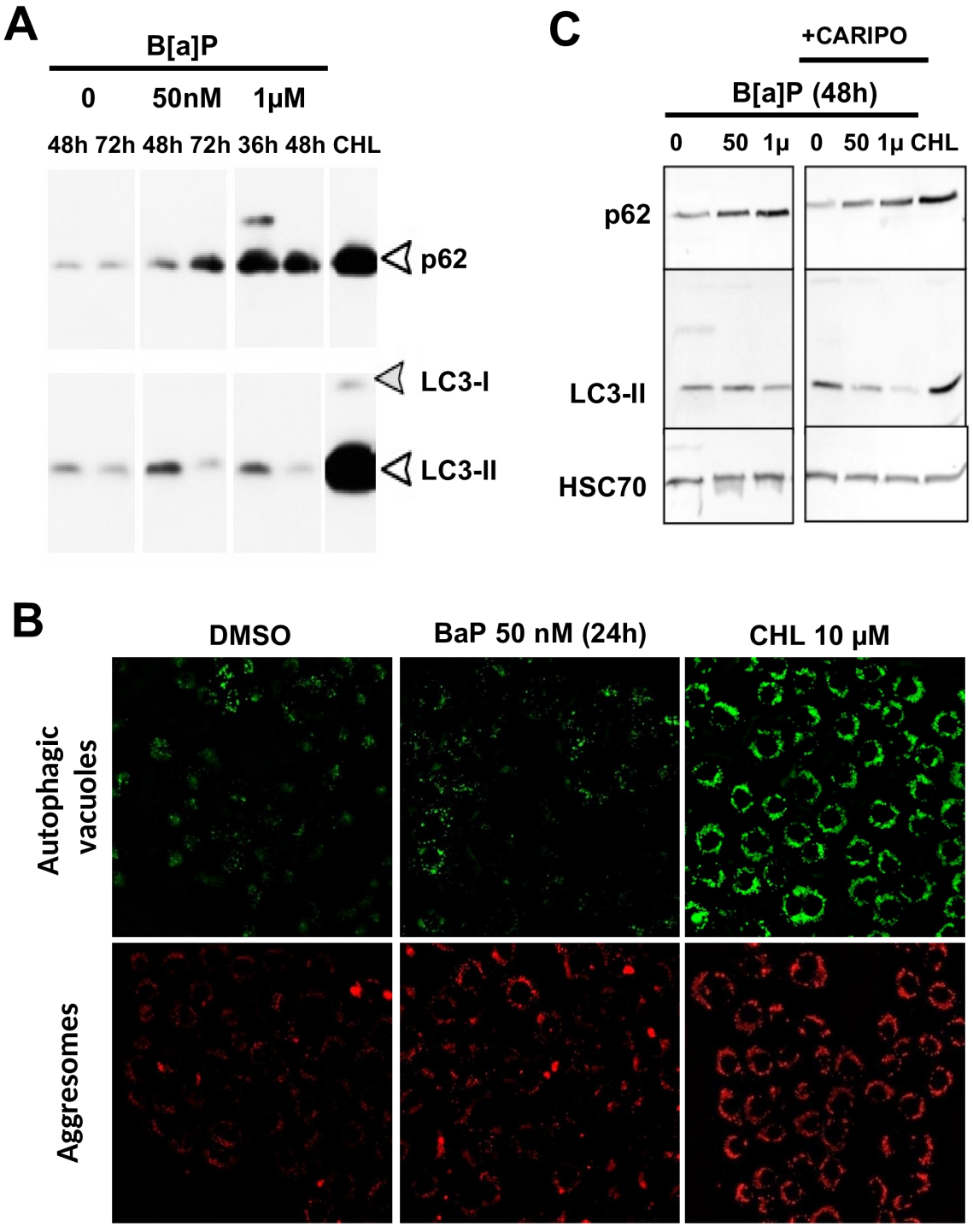
This table shows the different possible targets for changes in intracellular pH (pH_i) or extracellular pH (pH_e), as well as for changes in the pH of both mitochondrial matrix (pH_m) and lysosomes (pH_L) that would occur upon exposure to environmental carcinogens.

HKII: hexokinase II; ETC: mitochondrial electron transport chain ; IF1 : inhibitory factor 1 ; ECM : extracellular matrix; GPCR: G protein coupled receptor.

Alkaline pH_i	-membrane characteristics (fluidity) -activity of metabolic enzymes -protein interactions (HKII) -activity of F0F1-ATPase -gene expression …
Acid pH_m	-activity of ETC complexes (eg. complex II) -activation of IF1 …
Alkaline pH_L	-pH_i homeostasis -autophagy process -iron homeostasis -caspase activation through protease release …
Acid pH_e	-DNA repair -vesicle trafficking and release -activity of ECM enzymes -gene expression -miRNA expression -pH-sensitive GPCR activation ….


X
Phase 0
(uptake)
X
Phase 1
(oxidation)
O2
ROS
X*
damages
DNA
Proteins
Lipids
XOH
Phase 2
(conjugation)
ROH
XOR
Phase 3
(export)
XOR


Physiological conditions
pHm 8.0
pHL~4.5-5.0
pHe 7.4
NHE1
Resting pHi (~7.1-7.2)
B[a]P-exposure conditions
↓pHm (~6.5)
↑pHL
↓pHe
Na+
NHE1
H+
↑pHi (~7.4-7.5)


A
B[a]P
0
50nM
1μM
48h 72h 48h 72h 36h 48h CHL
p62
LC3-I
LC3-II
C
+CARIPO
B[a]P (48h)
0 50 1μ 0 50 1μ CHL
p62
LC3-II
HSC70
B
DMSO
BaP 50 nM (24h)
CHL 10 μM
Autophagic vacuoles
Aggresomes

HIGHLIGHTS

Benzo[a]pyrene, a well-known environmental carcinogen, alters H^+ dynamics.

NHE1 activation by B[a]P plays a key role in H^+ dynamics alterations.

B[a]P-altered pH controls cell death/survival balance via mitochondria and lysosome.

B[a]P impact on H^+ dynamics fits with the malignant tumor-related H^+ gradient reversal.

Altered H^+ dynamics might be universal mechanisms in environmental carcinogenesis.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: