Loss of Protein Kinase Cδ/HuR Interaction Is Necessary to Doxorubicin Resistance in Breast Cancer Cell Lines

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ABSTRACT

The protein kinase Cδ (PKCδ) interacts with and phosphorylates HuR, dictating its functionality. We show here that the genotoxic stimulus induced by doxorubicin triggers PKCδ interaction with HuR and leads to HuR phosphorylation on serines 221 and 318 and cytoplasmic translocation. This series of events is crucial to elicit the death pathway triggered by doxorubicin and is necessary to promote HuR function in post-transcriptional regulation of gene expression, because genetic ablation of PKCδ caused the inability of HuR to bind its target mRNAs, topoisomerase IIα (TOP2A) included. In vitro select doxorubicin-resistant human breast cancer cell lines upregulating the multidrug resistance marker ABCG2, PKCδ, and HuR proteins were coordinately downregulated together with the doxorubicin target TOP2A protein whose mRNA was HuR-regulated. Therefore, we show here that PKCδ, HuR, and TOP2A constitute a network mediating doxorubicin efficacy in breast cancer cells. The importance of these molecular events in cancer therapy is suggested by their being profoundly suppressed in cells selected for doxorubicin resistance.

Introduction

The efficacy of anticancer therapy is decreased by the onset of resistance mechanisms. This phenomenon holds true for classic cytotoxic drugs such as 5-fluorouracil, cisplatin, and anthracyclines (Broxterman et al., 2009) and for target-oriented anticancer agents such as tyrosine kinase inhibitors. It takes place through different mechanisms, including point mutation or amplification of the gene coding for the target enzyme and activation of alternative salvage pathways (Ercan et al., 2012). In the case of the anthracycline doxorubicin, cancer cells become refractory to therapy mainly by increasing expression of extrusion pumps such as the ABC transporters (Gros et al., 1986) and/or decreasing the expression level of the doxorubicin target enzyme topoisomerase II (Fry et al., 1991). HuR/ELAVL1 is a widely studied and ubiquitously expressed RNA-binding protein playing a pleiotropic role in cells and is involved in regulation of the major cell programs for proliferation, apoptosis, and differentiation, especially under cell stress conditions (Hinman and Lou, 2008). According to the cellular context, HuR binds to AU (adenine/uridine)-rich elements of primarily noncoding regions of mRNAs and of pre–mRNAs regulating their splicing, export, stability, and translation (Srikantan and Gorospe, 2012). Because HuR coordinates the maturation of thousands of mRNA species (Mukherjee et al., 2009; Dassi et al., 2013), it is not surprising that a deregulation of the activity of this protein is associated with cancer and inflammatory diseases (Pascale and Govoni, 2012; Srikantan and Gorospe, 2012). Recently, HuR has been addressed to mediate the efficacy of gemcitabine and doxorubicin because it post-transcriptionally regulates deoxycytidine (Costantino et al., 2009) and topoisomerase IIα (TOP2A) (Srikantan et al., 2011), the two enzymes, respectively, responsible for the effects of these drugs. It has been shown that HuR plays a role in promoting chemoresistance in glioblastoma multiforme by increasing the stability of the mRNA of the antiapoptotic gene BCL-2 (Filippova et al., 2011). We have reported the downregulation of HuR in doxorubicin-resistant breast cancer cell lines (Latorre et al., 2012). Post-translational modifications, such as phosphorylation (Eberhardt et al., 2012) and arginine methylation (Vázquez-Chantada et al., 2010), dictate HuR functional plasticity. Protein kinase Cδ (PKCδ) is among the proteins demonstrated to interact with HuR, and it has been shown to phosphorylate HuR in serines 218 and 321, thereby triggering its cytoplasmic translocation in human mesangial cells (Doller et al., 2010). In this way, PKCδ also sustains HuR aberrant functions in colon cancer cells (Doller et al., 2011). PKCδ belongs to the novel subgroup of PKCs and is regulated by diacylglycerol, but it is calcium-insensitive (Mellor and Parker, 1998). This kinase is activated by a number of proapoptotic stimuli, such as DNA-damaging agents and reactive oxygen species (Anantharam et al., 2002), both in cellular systems (Gonzalez-Guerrico and Kazanietz, 2005) and in mouse models (Leitges et al., 2001).
PKCδ, the c-Jun N-terminal kinase kinase, and caspase-2 are essential to mediate doxorubicin efficacy (Panaretakis et al., 2005; Lasfer et al., 2006) in primary acute lymphoblastic leukemia blasts and Hep3B cells, leading to the activation of the mitochondrial apoptosis pathway.

In this work, we identified PKCδ as a key player in HuR activation during doxorubicin-induced genotoxic stress, and by the evaluation of doxorubicin-resistant breast cancer cells, we found that the coordinated downregulation of PKCδ and HuR, with concomitant decreased expression of TOP2A enzyme, is preparatory to the onset of doxorubicin resistance.

Materials and Methods

Cell Lines. The MCF-7, MDA-MB-231, and SK-BR3 breast cancer cell lines, purchased from Interlab Cell Line Collection (Geona, Italy), were cultured in complete Dulbecco’s modified Eagle’s medium (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS; Lonza), 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (Lonza), or Opti-MEM (Lonza). MCF-7, MDA-MB-231, and SK-BR3 doxorubicin-resistant cells were derived from the respective parental cell lines by culturing under increasing doxorubicin concentrations, according to Latorre et al. (2012).

Chemicals, Antibodies, Small Interfering RNA, and Short Hairpin RNA. The following chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO): rottlerin (R5648, PubChem substance ID 24278679), doxorubicin hydrochloride (D1515, PubChem substance ID 24893465), and protein A–coated agarose beads. The following antibodies and small interfering RNAs (siRNAs) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-HuR (sc-71290), anti-PKCδ (sc-937), anti-Pan-Ac, and anti-pan-P antibodies.

Pull Down and Dot Blot. HuR mutant and wild-type plasmids were transiently transfected in MCF-7 cells treated or not with 10 μM doxorubicin for 3 hours after 48 hours of PKCδ silencing and 24 hours of starvation. Recombinant HuR protein was purified by affinity chromatography with HisTrap HP resin (GE Healthcare) and eluted with imidazole gradient ranging from 62.5 to 500 mM. Samples were analyzed by Western blotting.

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Results

Doxorubicin Triggers the Interaction between HuR and PKCδ. We previously showed that HuR was induced by doxorubicin to shuttle into the cytoplasm and was antagonized in the movement by the drug rottlerin. This drug was able to increase doxorubicin-induced HuR nuclear retention and prevent doxorubicin-induced HuR phosphorylation in breast cancer cells (Latorre et al., 2012). Despite the action of rottlerin being substantially unspecific (Soltoff, 2007), its first identified molecular target was PKCδ
Moreover, PKCδ has been reported to be involved in the regulation of HuR in different cell systems but not in the presence of a genotoxic stimulus. Therefore, to understand whether the inhibition of doxorubicin-induced phosphorylation of HuR by pretreatment with rottlerin could be explained by a direct involvement of PKCδ in the process, we investigated whether PKCδ was activated and induced to bind to HuR by doxorubicin. As shown in Fig. 1A, HuR and PKCδ coimmunoprecipitated in the presence of doxorubicin. PKCδ was scarcely present in the pellet derived from HuR immunoprecipitation in cells grown in media containing serum, and it was not detectable in cells grown under serum starvation. It is noteworthy that the interaction was strongly enriched following addition of doxorubicin to the medium. Moreover, pretreatment with rottlerin hampered doxorubicin-induced PKCδ and HuR coimmunoprecipitation. To assess whether PKCδ is pivotal in mediating doxorubicin-induced HuR phosphorylation, we performed siRNA-mediated silencing of PKCδ followed by a two-dimensional Western blotting against HuR. As shown in Fig. 1B, during PKCδ silencing, HuR is post-translationally modified in cells grown in serum, whereas it is not during starvation. Nevertheless, in the absence of PKCδ, treatment with doxorubicin reduced the number of HuR phosphorylations compared with the control condition (Fig. 1B), indicating that PKCδ is responsible for most of them during doxorubicin administration. Importantly, doxorubicin-induced HuR cytoplasmic accumulation was abolished (Fig. 1C) during silencing of PKCδ, and doxorubicin treatment induced a milder apoptotic response than in control conditions, as already observed (Panaretakis et al., 2005) (Fig. 1D). Therefore, PKCδ modulates the apoptotic response by inducing phosphorylation and regulating the cell localization of the RNA binding protein.

**Fig. 1.** PKCδ is involved in the apoptotic response to doxorubicin via complex formation with HuR. (A) HuR and PKCδ coimmunoprecipitate upon doxorubicin administration. Coimmunoprecipitation assessing the HuR and PKCδ protein-protein binding in the presence of 10 μM doxorubicin for 3 hours (Doxo), in standard growing conditions [fetal bovine serum (FBS)], in starvation (Starv), and in cotreatment with doxorubicin and rottlerin (Doxo-Rott). Immunoprecipitation samples, input (In), preimmune immunoglobulins immunoprecipitate (IgG), anti-HuR immunoprecipitate (HuR), and anti-PKCδ immunoprecipitate (PKCδ) were analyzed by Western blotting probing with anti-HuR or PKCδ antibody. HuR binds to PKCδ following doxorubicin treatment, and Rottlerin inhibits this interaction. (B) PKCδ silencing blocks doxorubicin-induced HuR phosphorylations. Two-dimensional Western blotting probed for HuR on MCF-7 cells treated with doxorubicin (10 μM for 3 hours) in the presence of PKCδ silencing (Si). In the lower part of the panel is a representative Western blotting anti-PKCδ showing the protein level in the different samples: standard growing (FBS), starvation (Starv), and doxorubicin treatment, and Rottlerin inhibits this interaction. (C) PKCδ silencing blocks doxorubicin-induced HuR phosphorylations. Two-dimensional Western blotting probed for HuR on MCF-7 cells treated with doxorubicin (10 μM for 3 hours) in the presence of PKCδ silencing (Si). In the lower part of the panel is a representative Western blotting anti-PKCδ showing the protein level in the different samples: standard growing (FBS), starvation (Starv), and doxorubicin treatment, and Rottlerin inhibits this interaction. (D) PKCδ silencing inhibits doxorubicin-induced HuR cytoplasmic translocation. Western blots on nuclear (n) and cytoplasmic (c) fractions of MCF-7 cells were probed for HuR. Starved MCF-7 cells (Starv) were treated with doxorubicin 10 μM for 3 hours (Doxo) or not (NTC) in the presence of PKCδ silencing (Si PKCδ). Probing for histone H3 (H3) and lactate dehydrogenase (LDH) was used as control for proper biochemical fraction separation. Silencing of PKCδ hampers doxorubicin HuR cytoplasmic translocation. (D) PKCδ silencing minimizes the doxorubicin-induced apoptotic response. *P < 0.05. Representative results out of at least three independent experiments; each experiment was run in technical triplicate. Number of cells was estimated to be identical in all of the experiments by measurement of ATP levels.
HuR, previously identified to mediate doxorubicin cell response upon insurgence of phosphorylations and cytosolic translocation (Latorre et al., 2012).

**PKCδ Mediates Doxorubicin Efficacy by Phosphorylating HuR on Serines 221 and 318.** The two HuR target residues of PKCδ have been endowed with different functions encompassing the ability to bind to RNA (Ser318) and the ability to shuttle between the nucleus and the cytoplasm (Ser221) (Doller et al., 2010; Schulz et al., 2013). To investigate PKCδ-mediated and doxorubicin-induced HuR phosphorylations more deeply, we generated two HuR mutants, HuR-S221A and HuR-S318A, that are nonphosphorylatable mutants by PKCδ (Doller et al., 2010). As shown in Fig. 2, upon starvation, the basal phosphorylation level of HuR was completely abolished, but doxorubicin was still able to induce phosphorylations in HuR in the ectopically expressed protein [wild-type HuR (WT-HuR)], as was the case for the endogenous counterpart (Fig. 1B). Silencing of PKCδ blocked doxorubicin-induced HuR phosphorylations, and since no further phosphorylations were detected in nonphosphorylatable mutants S221A and S318A, we can conclude that PKCδ mediates the doxorubicin-induced signaling cascade phosphorylating HuR on these two protein residues. Taken together, these results indicate that the interaction between PKCδ and HuR during doxorubicin administration causes phosphorylation in serines 221 and 318 of HuR (Fig. 2).

**PKCδ Modulates Doxorubicin-Induced HuR Post-transcriptional Response.** Because HuR is a major post-transcriptional regulator of stress-induced cell responses and regulates the post-transcriptional response to doxorubicin, we investigated whether PKCδ would affect HuR binding to specific target mRNA during doxorubicin administration. We chose three already validated targets (c-MYC, SOCS3, and FOS) that are susceptible to doxorubicin-induced HuR-enriched genes and already known to be targets of HuR: antiapoptotic genes MCL-1 and BCL-2 (Filippova et al., 2011). All of these transcripts were preferentially bound by HuR upon doxorubicin administration, but not after silencing of PKCδ (Fig. 3A). These data indicate that PKCδ affects HuR mRNA binding, allowing the association of HuR with specific target mRNA during doxorubicin administration. The HuR/mir-548c-3p post-transcriptional regulation of TOP2A has been indicated to be responsible for doxorubicin efficacy (Srikantan et al., 2011). During doxorubicin administration in starved cells, we could not detect TOP2A mRNA and the formation of a TOP2A mRNA/HuR protein complex (data not shown), knowing that TOP2A is expressed only during the G2/M phase (Srikantan et al., 2011). Concurrently with the increase of TOP2A mRNA expression during cell cycling, HuR bound to this mRNA, and after silencing of PKCδ and without doxorubicin, HuR lost this ability (Fig. 3B). Confirming the role of HuR in the post-transcriptional control of TOP2A mRNA, in stably HuR-silenced cells, TOP2A mRNA has a lower expression level compared with wild-type cells (Fig. 3C). Therefore, we can conclude that the protein-protein interaction between

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**Fig. 2.** HuR Ser221 and Ser318 were phosphorylated upon doxorubicin administration via PKCδ. Dot blot after pull down of ectopically transfected recombinant, His-tagged, HuR protein (rHuR). Empty vector, wild-type rHuR (wt), S221A, and S318A rHuR have been pulled down after transfection of scramble siRNA (scrm) or siPKCδ and in the presence or absence of doxorubicin treatment (10 μM for 3 hours). Membranes were probed with antibody anti-HuR to evaluate the rHuR precipitation, and with antibody antipan-phosphorylation to evaluate the level of rHuR phosphorylation. The experiments were run during starvation and exposure to doxorubicin for 4 hours. rHuR was phosphorylated during doxorubicin treatment and in the presence of PKCδ. Mutants of rHuR were not phosphorylated during doxorubicin treatment. Bottom panel: representative Western blot showing the level of silencing of PKCδ. Doxo, doxorubicin; NTA, nitrilotriacetic acid resin; NTC, nontreated control; siPKCd, small interfering RNA for PKCδ treatment.
PKC\(\beta\) and HuR, leading to HuR phosphorylation and cytosolic localization, is also responsible for the HuR post-transcriptional activity during doxorubicin-induced cell stress.

**PKC\(\beta\) Is Downregulated Together with HuR in Doxorubicin-Resistant Breast Cancer Cells.** Given the importance of PKC\(\beta\) in the cellular response to doxorubicin, we evaluated its expression level in two populations of doxorubicin-resistant breast cancer cells that we had previously developed (Latorre et al., 2012): MCF-7/doxorR and MDA-MD231/doxorR. In addition, we used one population that has been exposed continuously to doxorubicin for at least 1 month but failed to gain full doxorubicin resistance (SK-BR3/no-doxor), which therefore served as a negative control. As shown in Fig. 4A, in doxorR populations, PKC\(\beta\) was downregulated with respect to the parental doxorubicin-sensitive cells, as was HuR, whereas downregulation did not happen in the SK-BR3/no-doxorR population. Interestingly, TOP2A protein level decreased significantly in breast cancer-resistant cells that showed a low HuR expression level (Fig. 4A), consistent with what we observed in the stably HuR-silenced MCF-7 cells (Fig. 3C). Moreover, the ABCG2 transporter, a marker for the doxorubicin resistance phenotype, was found to be overexpressed, compared with parental cells, in the MCF-7/doxorR and in the MDA-MD231/doxorR strain but not in the SK-BR3/no-doxorR strain (Fig. 4B). We then performed rescue of function experiments by replenishing PKC\(\beta\) and WT-HuR in doxorR cell populations. As shown in Fig. 5A, ectopic expression of PKC\(\beta\) allowed recovery of the doxorubicin-induced apoptotic response in doxorR cells to the same extent that ectopic expression of HuR did. Interestingly, S221A-HuR and S318A-HuR mutants were not able to restart the apoptotic response, indicating that the phosphorylation of these two residues is necessary to activate HuR functionality. When we looked at the cell localization of exogenous HuR in doxorR cells and upon doxorubicin administration during starvation, we observed that, whereas WT-HuR translocated into the cytoplasm, S221A-HuR did not (Fig. 5C), suggesting that HuR can mediate doxorubicin efficacy upon PKC\(\beta\) interaction and subsequent translocation into the cytoplasm.
These results show that long-term exposure to doxorubicin causes the increase of the resistance mechanism via a variety of strategies, comprising the typical overexpression of the ABCG2 channel and the downregulation of proteins involved in mediating the apoptotic response (PKCd, HuR) by post-transcriptional regulation of downstream genes.

Discussion

Within this article, we showed that doxorubicin triggers the interaction of the proapoptotic kinase PKCd with the RNA-binding protein HuR by determining HuR post-translational modifications in Ser221 and Ser318, thus activating HuR post-transcriptional functionality, apoptotic response, and onset of pharmacoresistance. Doxorubicin requires both PKCd and HuR to fully exert its toxicity; PKCd is needed in Jurkat cells to activate caspase-2 (Panaretakis et al., 2005), whereas HuR, in MCF-7 cells, post-transcriptionally regulates the expression level of TOP2A (Srikantan et al., 2011). Here, we described the interplay among these factors, showing that the doxorubicin-dependent interaction between PKCd and HuR is functional to the doxorubicin-induced apoptotic cell response, because the loss of phosphorylation on HuR residues Ser221 and Ser318 is critical to elicit the full apoptotic response (Figs. 1D, 2, and 5A). These observations are in line with consolidated reports that describe the interplay between PKCd and HuR but in the absence of genotoxic stress (Doller et al., 2009, 2011). Moreover, the doxorubicin-induced, HuR-mediated, post-transcriptional response, comprising the binding of HuR to the mRNAs of antiapoptotic genes such as MCL-1 and BCL-2 and the doxorubicin target encoding TOP2A (Srikantan et al., 2011; Latorre et al., 2012), was lost upon PKCd silencing. We then evaluated whether doxorubicin sensitivity, PKCd/HuR mediated, could explain the onset of pharmacoresistance, at least in vitro. We previously reported that we had observed HuR downregulation in two different breast cancer cell line populations after prolonged exposure to increasing doses of doxorubicin as a single agent. Recently (Wang et al., 2013), it has been reported that HuR cytoplasmic accumulation, a synonym of high HuR activation, could not predict neoadjuvant chemotherapy sensitivity and efficacy in patients with primary breast cancer but suggested that high HuR cytoplasmic localization can be a negative predictor in the same patients. HuR cytoplasmic localization is therefore a strong indicator of the aggressiveness of the tumors and can be ascertained to the massive presence of antiapoptotic, mitogenic, and proliferative stimuli that HuR receives and mediates. From this perspective, HuR-linked pharmacoresistance due to overexpression of BCL-2 family members and sustained by HuR post-transcriptional activity was observed in glioblastoma cells that carried innate chemoresistance (Filippova et al., 2011), as could be the case for highly expressing HuR breast cancer patients. However, one should note the peculiar case of pancreatic adenocarcinoma sensitivity to the single agent gemcitabine, where high cytoplasmic HuR clearly stratified the patients with the best prognosis (Costantino et al., 2009). In this article, we also report that, by further characterization of our cell system and for the first time to our knowledge, in acquired doxorubicin-resistant breast cancer cell lines, downregulation of PKCd, HuR, and TOP2A proteins occurs simultaneously, as if resistant cells were corepressing a whole pathway responsible for doxorubicin efficacy. Indeed, the net result of HuR post-transcriptional...
PKCδ/HuR is a key interaction in doxorubicin-resistant cells. 

Fig. 5. PKCδ and HuR reactivate the doxorubicin-induced apoptotic response in MCF-7 doxorubicin-resistant (DR) cells. (A) Ectopic expression of PKCδ and HuR in MCF-7 doxorubicin-resistant (DR) reactivates the doxorubicin-induced apoptotic response. Histogram showing caspase-3 and -7 activation recorded as chemiluminescence in MCF-7 and MCF-7 DR cells in starvation (Starv) and after 4 hours of 10 μM doxorubicin (Doxo). DR cells were transfected with empty vector (--), PKCδ, WT-HuR, S221A, and S318A HuR mutants (S221A, S318A). *P < 0.05. Representative results out of at least three independent experiments; each experiment was run in technical triplicate. Number of cells was estimated to be identical in all of the experiments by measurement of ATP levels. (B) Ectopic expression of PKCδ in MCF-7 and MCF-7 DR. Representative Western blot of the expression level of PKCδ in MCF-7 and MCF-7 DR cells after ectopic expression of the kinase. The blot shows the decrease of the expression level of PKCδ in MCF-7 DR and the efficacy of the ectopic overexpression. (C) The S221A HuR mutant cannot shuttle into the cytosol during doxorubicin treatment in MCF-7 DR cells. Representative immunofluorescence of WT-HuR and S221A HuR mutant ectopically transfected in MCF-7 DR cells treated with doxorubicin. WT-HuR can shuttle into the cytoplasm and restart the doxorubicin-induced apoptotic response; S221A is confined to the nucleus and is not able to rescue HuR functionality. DAPI, 4,6-diamidino-2-phenylindole; NTC, nontreated control.

control is complex and depends on the cellular and experimental conditions and is possibly even more complicated in the clinical setting. In these resistant cell lines, we observed that doxorubicin activation of the PKCδ/HuR axis, leading to the early signaling cascade to apoptosis, is repressed and that the rescue of the apoptotic response upon ectopic expression of PKCδ is evidence of its importance of the response. At the same time, inhibition of the PKCδ/HuR-dependent post-transcriptional regulation process leads, as reported, to the down-regulation of the doxorubicin target TOP2A and to the impairment of the HuR-mediated control on other downstream genes. Therefore, the PKCδ/HuR axis is a key pivotal factor in the doxorubicin-induced activity and pharmacoresistance in breast cancer cell lines.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Lattore, Quattrone, Provenzani.

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