Metformin Induces Both Caspase-Dependent and Poly(ADP-ribose) Polymerase-Dependent Cell Death in Breast Cancer Cells

Yongxian Zhuang¹ and W. Keith Miskimins¹,²,*

¹Cancer Biology Research Center, Sanford Research/USD, Sioux Falls, South Dakota

²Department of Obstetrics and Gynecology and Division of Basic Biomedical Sciences, Sanford School of Medicine of the University of South Dakota, Sioux Falls, South Dakota

*Corresponding author

Address: Cancer Biology Research Center, Sanford Research/USD, 2301 East 60th Street-North, Sioux Falls, SD 57104, USA

Email: Keith.Miskimins@usd.edu
Telephone: 605-328-0464

Grant Support: US Public Health Service, National Cancer Institute, R01CA084325 (W.K. Miskimins) and Susan G. Komen for the Cure grant KG100497 (W.K. Miskimins).

Running Title: Metformin cytotoxicity to breast cancer cells

Key words: metformin, breast cancer, apoptosis, caspases, PARP, AIF
Abstract

There is substantial evidence that metformin, a drug used to treat type 2 diabetics, is potentially useful as a therapeutic agent for cancer. However, a better understanding of the molecular mechanisms through which metformin promotes cell cycle arrest and cell death of cancer cells is necessary. It will also be important to understand how the response of tumor cells differs from normal cells and why some tumor cells are resistant to the effects of metformin. We have found that exposure to metformin induces cell death in all but one line, MDA-MB-231, in a panel of breast cancer cell lines. MCF10A non-transformed breast epithelial cells were resistant to the cytotoxic effects of metformin, even after extended exposure to the drug. In sensitive lines, cell death was mediated by both apoptosis and a caspase-independent mechanism. The caspase-independent pathway involves activation of poly(ADP-ribose) polymerase (PARP) and correlates with enhanced synthesis of poly(ADP-ribose) and nuclear translocation of AIF, which plays an important role in mediating cell death. Metformin-induced PARP-dependent cell death is associated with a striking enlargement of mitochondria. Mitochondrial enlargement was observed in all sensitive breast cancer cell lines but not in non-transformed cells or resistant MDA-MB-231. Mitochondrial enlargement was prevented by inhibiting PARP activity or expression. A caspase inhibitor blocked metformin-induced apoptosis but did not affect PARP-dependent cell death or mitochondrial enlargement. Thus metformin has cytotoxic effects on breast cancer cells through two independent pathways. These findings will be pertinent to efforts directed at using metformin or related compounds for cancer therapy.
Introduction

Metformin is a drug that is commonly prescribed to treat type 2 diabetics. Recently, metformin has received attention as a potentially useful therapeutic agent for treating cancer. Population studies indicate that type 2 diabetics taking metformin have a significantly reduced risk of cancer and lower cancer-related mortality than diabetics not taking metformin (1-3). For diabetic patients with breast cancer, it was demonstrated that patients on metformin had a significantly better response to neoadjuvant chemotherapy than patients not taking metformin (4). Preclinical animal model systems have demonstrated reduced tumor growth with metformin for colon (5, 6); breast (7-9), pancreatic (10), and lung (11) cancers. One study using a rat model of chemically induced mammary carcinogenesis found no significant effects of metformin on tumor growth (12). Another study using xenografts of the human MDA-MB-435 cell line, now thought to be derived from melanoma (13), observed increased tumor growth with metformin treatment.

The mechanisms by which metformin produces its inhibitory effects on cancer development and tumor growth are not completely understood. These could be through indirect effects on systemic levels of insulin or glucose (14, 15), or through direct effects on tumor cell growth and survival. Direct effects of metformin on cancer cells include inhibition of cell proliferation (6, 9, 10, 16-22) and induction of cell death (5, 8, 9, 18, 19, 23). Inhibition of cancer cell proliferation in response to metformin appears to involve activation of AMP-activated protein kinase (AMPK) (6, 9, 10, 17-19, 21, 22), inhibition of mTOR activity and protein translation (17), and downregulation of cyclin D1 leading to cell cycle arrest in G1 (6, 9, 16, 22). In those studies where metformin has been shown
to promote cell death the mechanism appears to involve activation of apoptotic pathways (5, 9, 19, 24). In a colon cancer model system, metformin-stimulated apoptosis was specifically associated with loss of p53-dependent enhancement of autophagy and glycolysis and was stimulated by nutrient deprivation (5). In other culture systems metformin displayed enhanced cytotoxicity in combination with glucose deprivation (23, 25), cisplatin (18), doxorubicin (8, 26), or buthionine sulfoximine (26).

Based on recent epidemiological, clinical, and preclinical data there is growing interest in the potential use of metformin for treating cancer (27). In this regard, a better understanding of the molecular mechanisms and signaling pathways through which metformin promotes cell cycle arrest and cell death of cancer cells is needed. It will be important to determine how the response of tumor cells differs from normal cells and why some tumor cells are resistant to the effects of metformin. In this study we have examined metformin-induced cell death in a panel of breast cancer cell lines. All but one breast cancer cell line underwent cell death in response to metformin. Non-transformed breast epithelial cells were also resistant to the cytotoxic effects of metformin. In sensitive cell lines, cell death was mediated by both caspase-dependent and caspase-independent mechanisms. The caspase-independent pathway involved activation of poly(ADP-ribose) polymerase (PARP), was associated with mitochondrial enlargement, and was reduced by depletion of AIF.

Materials and Methods

Chemicals and Cell culture. Metformin (1,1-dimethylbiguanide) was purchased from Sigma Chemical, PARP inhibitor II (INH2BP, 5-Iodo-6-amino-1,2-benzopyrone)
was purchased from Calbiochem, and caspase inhibitor (Q-Val-Asp-OPh) was purchased from MP Biomedicals. Caspase Inhibitor sample pack (FMKSP01) was purchased from R&D systems. The cell lines MCF7, T47D, MDA-MB-453, BT474, MDA-MB-231, and MCF10A cells were purchased from American Type Culture Collection (ATCC). ATCC cell lines are authenticated by STR analysis. Upon receiving the cell lines they were immediately cultured and expanded to prepare frozen ampule stocks. Cells were passaged for no more than 2-3 months before establishing new cultures from the early passage frozen ampules. The stable cell lines MCF7-shPARP and MCF7-shLuc were obtained from Dr. W. Lee Kraus (Cornell University) and have not been authenticated. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator with 5% CO₂.

**Trypan blue exclusion assay.** Cells were plated in 35 mm dishes. After treatment as indicated in each figure, cells were harvested by trypsinization and stained using 0.02% trypan blue. Trypan blue positive and negative cells were counted using a hemacytometer.

**Confocal microscopy.** Cells were treated as indicated in each figure. Cells were washed in phosphate buffered saline (PBS) and fixed in 4% paraformaldehyde for 15 minutes. For detection of transfected fluorescent proteins, cells were then washed in PBS and mounted in Vectashield medium containing 4, 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). For detection of endogenous proteins by immunofluorescence, cells were permeabilized in 0.25% Triton X-100 for 5 minutes and then washed in PBS three times. This was followed by blocking in 10% bovine serum
albumin (BSA) in PBS for 30 minutes and then incubation in primary antibody for 2 hours at 37°C. Primary antibody (1:100) was prepared in 3% BSA in PBS. Slides were washed three times in PBS and incubated with Alexa Fluor 594-labeled secondary antibody (1:500, Molecular Probes) for 45 minutes. Finally, slides were washed in PBS three times and mounted using Vectashield medium containing DAPI. Slides were observed using an Olympus FV1000 confocal microscope. The following primary antibodies were used: AIF antibody (#4642, Cell Signaling, USA) and PAR antibody (#550781, BD Pharmingen, USA). Plasmids encoding mitochondrial targeted fluorescent proteins, pAcGFP1-Mito and pDsRed2-Mito, were purchased from Clontech.

**Annexin V/propidium iodide staining.** Cells were plated in 35 mm dishes and treated as indicated for 3 days. Cells were washed once in PBS and stained using the ApoAlert Annexin V-FITC Apoptosis Kit (Clontech Laboratories) as follows. Cells were washed with Binding Buffer and then 200 μl of Binding Buffer containing 5 μl of propidium iodide (PI) and 10 μl Annexin V-FITC was added. The cells were incubated for 10 minutes in the dark. The cells were observed by fluorescence microscopy using a dual filter set for FITC and rhodamine.

**Electroporation.** Cells were plated into 150 mm dishes and cultured in DMEM with 10% fetal bovine serum for 2 days. Cells (2 x10⁶) were trypsinized and pelleted at 90 xg for 10 minutes. The supernatant was completely discarded and the cells were resuspended at room temperature in Cell Line Nucleofector Solution V (Amaxa) to a final concentration of 2 x10⁶ cells per 100 μl. The cell suspension was mixed with 2 μg DNA for each sample. The sample was transferred into an Amaxa cuvette and cells were electroporated using a Nucleofector II electroporation apparatus with program E-
The cells were transferred from the cuvette into 35 mm dishes containing warm medium. Medium was changed after overnight incubation. Stably transfected cells were selected using 400 μg/ml G418 disulfate (Sigma, USA).

**Inhibition of AIF expression using siRNAs.** On-TARGET Plus Smart pool siRNA targeting AIF and non-targeting siRNA were purchased from Thermo Scientific. MCF7 cells that were ~30% confluent were transfected with the siRNAs using Lipofectamine 2000 (Invitrogen). One day after transfection the cells were treated with either DMSO or caspase inhibitor (10 μM) with or without metformin (8 mM) for three days. Cells were trypsinized and trypan blue exclusion assays were used to count dead and live cells.

**Western blotting.** Cells in 35 mm dishes were rinsed with PBS and lysed by addition of 1X sodium dodecylsulfate (SDS) sample buffer [2.5 mM Tris-HCl, (pH 6.8), 2.5% SDS, 100 mM dithiothreitol, 10% glycerol, 0.025% pyronine Y]. Equal amounts of protein were separated on a 10% or 15% SDS-polyacrylamide gel. Proteins were transferred to Immobilon P membranes (Millipore) using a Bio-Rad Trans-blot apparatus with a transfer buffer of 48 mM Tris-HCl and 39 mM glycine. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline [10 mM Tris-HCl (pH 7.5), 150 mM NaCl] containing 0.1% Tween-20 (TBS-T) for 15-60 minutes at room temperature. The membrane was then incubated with the appropriate antibody in TBS-T containing 5% non-fat dry milk for 1 hour at room temperature or overnight at 4°C. After extensive washing in TBS-T the membrane was incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. After extensive washing in TBS-T, proteins were detected using the Super Signal West Pico chemiluminescent substrate.
(Pierce Biochemical). Anti-β-actin monoclonal antibody (1:10,000) was purchased from Sigma Chemical. Antibodies to PARP (#9542, used at 1:1000), cleaved caspase 7 (#9491, used at 1:1000) and AIF (#4642, used at 1:1000) were purchased from Cell Signaling. Secondary horseradish peroxidase-linked anti-mouse and anti-rabbit IgG antibodies (used at 1:5000) were purchased from Pierce Biochemical.

**Transmission electron microscopy.** Cells were treated with or without metformin for 2.5 days, trypsinized, and pelleted by centrifuging for 5 minutes at 1000 rpm in a MicroCL17R centrifuge. They were then washed in PBS and pelleted at 1000 rpm for 5 minutes. Cell pellets were fixed using 4% formaldehyde-1% glutaraldehyde. Cells remained in the fixative for a minimum of six hours. The pellet was rinsed three times with PBS for 5 minutes and the pellet was post fixed with buffered 1% osmium tetroxide for no less than one hour and no more than two hours. The pellet was rinsed with distilled water three times for 5 minutes each. Then it was dehydrated by rinsing sequentially in 50% acetone, 70% acetone, and 95% acetone, twice each, for 5 minutes. Then the pellet was immersed in 100% acetone for ten minutes. This was repeated three times. Infiltration was continued as follows. The dehydrated pellet was immersed into one part catalyzed resin to two parts acetone for a minimum of thirty minutes, two parts catalyzed resin to one part acetone for a minimum of sixty minutes, and 100% catalyzed resin for a minimum of one hour. The infiltrated pellet was embedded into catalyzed resin, and blocks were cured for eight hours in a 60-65°C oven. Thick sections were cut from the capsule and then one micron sections were cut. The last good section cut from the block was floated on distilled water. Then the sections were stained with toluidine blue. Thin sections were processed using a
Reichert-Jung Ultracat E Ultramicrotome then double stained using uranyl acetate (saturated uranyl acetate in 50% ethanol) and 2% lead citrate. Specimens were observed using a JEOL 1210 transmission electron microscope at 8000X or 15000X magnification.

**Flow cytometry.** MCF7 cells were treated with or without metformin for 3 days and then processed using the ApoAlert Annexin V-FITC Apoptosis kit (Clontech Laboratories). Cells were trypsinized and then pelleted by centrifugation. The cell pellet was washed in 1X PBS and then in 1X binding buffer. The cells were pelleted again and resuspended in 200 μl 1X binding buffer. Annexin V (5 μL) and PI (5 μL) were added and the cell suspension was incubated for 10 minutes in the dark. All flow cytometry measurements were performed using an Accuri C6 flow cytometer.

**Results**

**Metformin induces cell death in most breast cancer cells but not non-transformed mammary epithelial MCF10A cells.** Previously we treated MCF7 cells with metformin for 1 to 2 days, and found that MCF7 cell proliferation was inhibited (22). With an extended time of treatment many cells detach from the dish and appear to be dead. To examine this further, live cell numbers were measured using a trypan blue exclusion assay (Fig. 1A). Over the first two days of metformin treatment MCF7 cells were inhibited from proliferating as compared to controls, but by the third day the cell number started to decrease due to increased cell death. To determine if the cytotoxic effects of metformin are limited to MCF7 cells, we treated a panel of breast cancer cell lines with the drug. Live and dead cells were counted and the percentage of dead cells
was determined (Fig. 1B). T47D, MDA-MB-453, BT474, in addition to MCF7, underwent cell death in response to metformin. The breast cancer cell line MDA-MB-231 was resistant to the drug and showed almost no increase in cell death. The non-transformed human mammary epithelial cell line, MCF10A, was also resistant to the cytotoxic effects of metformin. MCF7 and T47D were the most sensitive of the breast cancer cell lines as indicated by the greater number of dead cells and shorter time to cell death. Even with extended treatment times no significant cell death was observed in metformin treated MDA-MB-231 or MCF10A cells (data not shown). MCF10A cells remained viable in the presence of metformin for up to two weeks, although proliferation was reduced relative to untreated cultures (data not shown). These data suggest that metformin promotes cell death in most breast cancer cells but not in normal mammary epithelial cells.

**Cell death induced by metformin has features of apoptosis.** In order to define the type of cell death caused by metformin in MCF7 cells, western blotting was performed to detect cleaved caspase 7, cleaved PARP1 and PARP1. MCF7 cells do not express caspase 3, but the executioner caspase 7 has previously been shown to be activated downstream of mitochondria-mediated apoptosis in this cell line (28). PARP1 is a substrate for both caspases 3 and 7 and cleaved PARP1 is a hallmark of caspase-dependent apoptosis. Cleaved caspase 7, the active form of the enzyme, was detected by the third day after metformin treatment and paralleled an increase in PARP1 cleavage (Fig. 2A). This suggests that an apoptotic cell death pathway is stimulated by metformin. To further explore this, MCF7 cells were treated with metformin and Annexin V-FITC/PI staining was performed (Fig. 2B). Annexin V-FITC staining indicates that
cells are dying through apoptosis while PI staining indicates that the cells have damaged plasma membranes and are dead. After metformin treatment for 3 days there is an increase in the number of cells that are Annexin V positive and PI negative, as well as an increase in the number of cells that are positive for both. Quantification by flow cytometry indicates that 23.6% of the cells are Annexin V positive in metformin treated cultures compared to 2.27% in control cultures (Fig. 2B, right panel). To confirm caspase-dependent apoptotic cell death, MCF7 cells were co-treated with an irreversible broad spectrum caspase inhibitor (Q-Val-Asp-OPh, 10 μM) and metformin for approximately 2.5 days and cells were harvested for western blotting. The pan-caspase inhibitor completely blocked activation of caspase 7 and cleavage of PARP in the metformin treated cells (Fig. 2C). Interestingly, we routinely observe a decrease in both full-length PARP and caspase 7 when cells are incubated with metformin and caspase inhibitors. This decrease does not correspond to an increase in cleaved forms of the protein but may be related to activation of AMPK and subsequent inhibition of mTOR, which leads to decreased protein synthesis. Metformin-induced activation of caspase 7 was also blocked by inhibitors that are specific for caspases 3/7 and 9, but not by an inhibitor of caspase 8 (Fig. S1A). The effect of the pan-caspase inhibitor on metformin-induced cell death was also monitored (Fig. 2D). MCF7 cells were co-treated with metformin and the pan-caspase inhibitor for 2.5 days and then the number of live and dead cells in the cultures was determined (Fig. 2D, left panel). Cell death in metformin treated cells was almost completely blocked by the presence of the caspase inhibitor at this time point, indicating that cells are dying through caspase-dependent apoptosis.
Even though inhibition of caspases was effective at blocking cell death at 2.5 days, extended incubation with metformin led to cell death regardless of the presence of Q-Val-Asp-OPh (Fig. 2D, right panel). In fact, there was more cell death in the present of the pan-caspase inhibitor at this time point. These results suggest that a second, caspase-independent, mechanism of cell death is promoted by metformin in this breast cancer cell line. Therefore, further experiments were performed to explore other potential mechanisms of cell death in metformin treated breast cancer cells.

**Metformin causes significant morphological changes in mitochondria that correlate with sensitivity to the drug.** Treatment of MCF7 cells with metformin leads to a striking accumulation of large, clear vesicles that are noticeably visible by phase contrast microscopy (Fig. 3A). These vesicles are observed in all of the metformin-sensitive breast cancer cell lines that have been examined (T47D, MDA-MB-453, BT474) but not in MDA-MB-231 or MCF-10A cells, which are resistant to the cytotoxic effects of the drug (Fig. 3A). The vesicles accumulate throughout the cytosol but appear to be most dense around the nucleus. To determine the origin of the vacuoles, transmission electron microscopy (TEM) was used. MCF7 cells were treated with metformin for 3 days and then harvested for TEM analysis (Fig. 3B). Compared to control cells, numerous enlarged organelles are observed in metformin treated cells. These organelles retain features of mitochondria, including clearly defined cristae (Fig. 3B). These results suggest that the vesicles observed by phase contrast microscopy are actually enlarged mitochondria. To confirm this, MCF7 cells were transfected with a construct encoding DsRed-Mito. This construct encodes a red fluorescent protein that is specifically targeted to mitochondria. After transfection, cells were treated with
metformin for 2.5 days and then observed by confocal microscopy. Metformin treatment causes significant morphological changes in DsRed-stained mitochondria (Fig. 3C). The mitochondria become enlarged and the DsRed-Mito signal becomes diffuse. Together these results show that metformin promotes morphological changes in mitochondria, including a dramatic enlargement and loss of internal structure. Since these mitochondrial changes are associated with sensitivity to metformin-induced cytotoxicity, we further explored their relationship to cell death mechanisms.

**Metformin induces PARP-dependent cell death that correlates with mitochondrial enlargement.** A second form of cell death that is associated with mitochondrial changes, but is caspase-independent, is PARP-dependent cell death. This form of cell death is distinguishable from apoptosis, autophagic cell death, and necrosis (29). It is initiated by overactivation of PARP and synthesis of its product poly(ADP-ribose) (PAR). PAR signals mitochondria to release AIF, which translocates to the nucleus to facilitate cell death. Previously, it was shown that AIF release mediates delayed onset cell death when the caspase-dependent pathway is blocked (30). We therefore examined the possibility that PARP-dependent cell death is initiated in metformin-treated breast cancer cells. MCF7 cells were treated with metformin for 4 days in the presence or absence of a chemical inhibitor of PARP and then the percentage of dead cells in the culture was determined (Fig. 4A, left panel). Inhibition of PARP led to a significant decrease in metformin-induced cell death. The effect of the PARP inhibitor is even greater when the cells are also treated with a pan-caspase inhibitor. This was further examined in a time course experiment in which the effects on metformin-induced cell death by the pan-caspase inhibitor, PARP inhibitor, or both
together was examined (Fig. S1B). The PARP inhibitor, alone, does not significantly reduce cell death until 3.5 days; whereas the pan-caspase inhibitor is effective at 1.5 and 2.5 days but not at 3.5 days. These results support the conclusion that metformin induces both PARP-dependent and caspase-dependent apoptotic cell death and that the PARP-dependent mechanism is delayed relative apoptosis.

To confirm results obtained using the chemical inhibitor of PARP, a cell line (MCF7-shPARP1) in which PARP1 expression is stably repressed via an shRNA was used (31). A cell line expressing an shRNA targeted to luciferase (MCF7-shLuc) was used as a control. MCF7-shLuc responds to metformin in a manner very similar to the parental cell line. After 2 days a significant number of dead cells were observed in the metformin-treated culture (Fig. 4B, left panel). Cell death corresponded to activation of caspase 7, the appearance of cleaved PARP1, and was substantially blocked by inclusion of the pan-caspase inhibitor (Fig. 4B, 4C). Two days treatment of MCF7-shPARP1 also led to substantial cell death but this was reduced compared to control cells. Following three days of metformin treatment, inhibition of caspases no longer blocked cell death in the control MCF7-shLuc cells but almost completely blocked cell death in MCF7-shPARP1 (Fig. 4B, right panel). Metformin induced activation of caspase 7 in MCF7-shPARP1 and this was also completely blocked by the pan-caspase inhibitor (Fig. 4C). These results further support the conclusion that metformin induces caspase-dependent apoptotic cell death as well a delayed form of cell death that is PARP-dependent.

Since cell death in response to metformin correlates with mitochondrial enlargement, we wanted to determine if this is associated with apoptosis, PARP-
dependent cell death, or both. MCF7 cells were treated with metformin in the presence or absence of caspase inhibitor, PARP inhibitor, or both and then examined by phase contrast microscopy (Fig. 5A). Inhibition of caspases had no effect on the formation of enlarged mitochondria that appear as phase transparent "vacuoles" in the cytoplasm. In contrast, inclusion of the PARP inhibitor almost completely blocked formation of the vacuoles. These results were confirmed using MCF7 cells stably expressing mitochondrial-targeted pAcGFP1 (Fig. 5B). In addition, MCF7-shLuc and MCF7-shPARP1 cells were treated with metformin in the presence and absence of caspase inhibitor and then observed by phase contrast microscopy. No enlarged mitochondrial were observed in MCF7-shPARP1 cells under any conditions (Fig. 5C). MCF7-shLuc cells behaved exactly like the parental cell line and enlarged mitochondrial appeared after treatment with metformin even in the presence of caspase inhibitor. It can be concluded that mitochondrial enlargement is specifically associated with PARP1 activation and PARP-dependent cell death.

Metformin treatment of sensitive breast cancer cells leads to enhanced PAR synthesis and release of AIF from mitochondria. PARP1 is a nuclear enzyme that catalyzes the synthesis of PAR. PAR has been shown to be an important signaling component of PARP-dependent cell death. Since metformin stimulates a delayed cell death response that is blocked by chemical inhibitors of PARP, or by siRNA-mediated repression of the enzyme, we examined various aspects of the PARP-dependent cell death pathway. Metformin-sensitive MCF7 cells or metformin-resistant MDA-MB-231 cells were treated with the drug for 2.5 days. PAR, the product of PARP, was then detected using immunofluorescence (Fig. 6A, B). In MCF7, PAR was not detectable in
untreated control cells but was induced by metformin (Fig. 6A, left panel). Induction of PAR synthesis by metformin was almost completely blocked by co-treatment with a PARP inhibitor (Fig. 6B). For MDA-MB-231 there was no difference in PAR levels between control and metformin treated cells (Fig. 6A, right panel). Thus PARP activation in response to metformin is limited to cells that undergo cell death when treated with the drug.

AIF, another key mediator of PARP-dependent cell death, is normally localized to the intermembrane space of mitochondria. PAR promotes translocation of AIF from mitochondria to the nucleus, causing DNA condensation and fragmentation, and subsequent cell death in a caspase-independent manner (32). To examine effects on AIF localization, MCF7 cells were treated with metformin, with or without PARP inhibitor, and then AIF was detected using immunofluorescence (Fig. 7A). In control cells, as expected, the pattern of AIF staining is consistent with localization to mitochondria. Metformin caused mitochondria to enlarge and AIF was excluded from these mitochondria. Release of AIF and mitochondrial enlargement in metformin treated cells was inhibited by co-treatment with PARP inhibitor (Fig. 7A). Nuclear uptake of AIF is a key event in PARP-dependent cell death. To examine this, MCF7 cells were treated with and without metformin for three days. Nuclear extracts were prepared and AIF was detected by western blotting (Fig. 7B). In metformin treated cells AIF was clearly present in the nuclear fraction but almost undetectable in control cells. To determine if AIF plays a role in metformin-induced cell death, MCF7 cells were transfected with a control siRNA or an siRNA targeted to AIF. In cells transfected with the AIF-targeted siRNA, expression of the protein was reduced by ~65% (Fig. 7C) and metformin-
induced cell death was significantly reduced (Fig. 7D). The inhibition of cell death in cells with reduced AIF expression was even greater when cells were also treated with pan-caspase inhibitor. Thus AIF plays an important role in the metformin-induced caspase-independent cell death mechanism that functions downstream of PARP activation.

Discussion

Metformin has previously been shown to inhibit proliferation of various types of cancer cells (6, 9, 10, 16-22). In addition, metformin has been shown to promote cell death of some cancer cells through activation of apoptotic pathways (5, 9, 19, 24). The data presented here confirm metformin-induced killing of breast cancer cells through apoptosis. Buzzai et al. (5) showed that metformin-stimulated apoptosis of colon cancer cells was associated with loss of p53-dependent enhancement of autophagy and glycolysis and that cell killing was stimulated by nutrient deprivation. In contrast, Ben Sahra et al. (33) showed that, in the presence of 2-deoxyglucose, metformin promotes apoptosis of prostate cancer cells in a p53-dependent manner. For breast cancer cells, p53 status does not seem to govern sensitivity to metformin. The cell lines T47D, MDA-MB-453, BT474, and MCF7 are all sensitive to the cytotoxic effects of metformin. MCF7 expresses wild-type p53 while all the other lines express mutated p53. MDA-MB-231, which continues to grow at concentrations that kill the other breast cancer cell lines, also expresses mutated p53.

A novel finding of the work presented here is that metformin induces a second form of cell death in breast cancer cells. This mode of cell death is dependent on PARP but
independent of caspase activity. It is blocked by chemical inhibition of PARP enzymatic activity and does not occur at significant levels in cells where PARP1 expression is repressed by shRNAs. PARP-mediated cell death has been observed under a number of different circumstances, including treatment with DNA alkylating agents, following oxidative DNA damage, treatment with neurotoxic compounds, and ischemia (30, 32, 34-47). PARP-dependent cell death appears to be a unique form of programmed cell death that is distinct from apoptosis, autophagic cell death, or necrosis (29). It was recently named parthanatos to distinguish it from the other forms of cell death (48). Key events in this cell death pathway include activation of PARP, extensive synthesis of PAR, signaling of PAR to the mitochondria, release of AIF from mitochondria, and nuclear uptake of AIF (29). Here we show that metformin treatment of MCF7 cells enhances both PAR synthesis and AIF uptake into the nuclear compartment. Reduction of AIF expression also reduced metformin-induced cell death. Elevated PAR synthesis is not observed in MDA-MB-231 cells which are resistant to the cytotoxic effects of metformin.

Hyperactivation of PARP also may cause cell death by depleting cellular NAD$^+$ levels. Since NAD$^+$ is required for both glycolysis and oxidative metabolism in mitochondria, depletion could lead to cellular energy collapse and subsequent cell death. Such a mechanism has previously been described for cell death in response to alkylating agents (32, 47) and has been referred to as “metabolic catastrophe” (49). It may be that cancer cells are more sensitive than normal cells to energy collapse because of high energy requirements for unrestricted proliferation. Depletion of cellular levels of NAD$^+$ may also affect cell survival through mechanisms other than energy...
collapse. Alano et al. (50) showed that addition of NAD$^+$ to the culture medium was able to block PARP-dependent translocation of AIF to the nucleus of astrocytes treated with DNA damaging agents. Overexpression of nicotinamide phosphoribosyltransferase (NAMPT), the rate limiting enzyme in the synthesis of NAD$^+$ from nicotinamide, protects cells from cell death in response to genotoxic compounds (51). This protective effect requires mitochondrial sirtuins, SIRT3 and SIRT4. It is possible that sensitivity to the cytotoxic effects of metformin are related to the cellular levels of NAMPT or sirtuins and it will be of interest to compare the levels and activity of these enzymes in sensitive and resistant cell lines.

PARP-dependent cell death of metformin treated cells appears to be delayed relative to apoptotic cell death and is associated with changes in mitochondrial morphology. In all metformin sensitive breast cancer cell lines examined, mitochondria become enlarged and this is blocked by PARP inhibitors but not by caspase inhibition. Metformin-induced mitochondrial enlargement was also inhibited by genetically repressing PARP1 expression in MCF7 cells. Inhibition of PARP blocks mitochondrial enlargement but does not prevent metformin-induced apoptotic cell death. Thus mitochondrial enlargement is specifically associated with the PARP-dependent cell death pathway in response to metformin. PARP-dependent cell death has previously been associated with the mitochondrial permeability transition (MPT)(50). MPT causes mitochondria to become permeable to small molecules and this leads to osmotic swelling of the organelles. MPT is inhibited by cyclosporin A or bongkrekic (52, 53) but we have been unable to prevent metformin-induced mitochondrial enlargement with either of these reagents (data not shown). Previously dos Santos et al. (54) observed
cyclosporine A-insensitive MPT induction that was associated with complex I inhibition. At present it is not clear what process leads to mitochondrial enlargement following metformin treatment or how this relates to PARP activation.

Metformin is known to be taken up and concentrated in mitochondria where it inhibits complex I of the electron transport chain (55). This leads to loss of mitochondrial membrane potential and inhibition of ATP production. Inhibition of complex I also promotes superoxide production within the mitochondrial matrix which could cause damage to mitochondrial proteins, lipids, and nucleic acids. These effects may lead to apoptotic cell death. However, it is difficult to connect them directly to PARP-dependent cell death. PARP1 is primarily a nuclear protein that is activated by DNA damage. Therefore, one possibility is that metformin-induced superoxide production within the mitochondrial matrix leads to accumulation of reactive oxygen species throughout the cell, causing nuclear DNA damage, PARP activation, PAR synthesis, and signaling back to mitochondria to promote AIF release and cell death. Another possibility is that mitochondrial-localized PARP1 is a key enzyme in metformin-induced cell death. There is substantial evidence that PAR synthesis takes place in mitochondria and that a portion PARP1 localizes to this organelle (56-62). Du et al. (57) showed that cell death induced by oxidative stress involves mitochondrial PARP1. Inhibition of mitochondrial PARP1 prevented NAD⁺ depletion within mitochondria, loss of mitochondrial membrane potential, release and nuclear uptake of AIF, and reduced cell death. It is therefore possible that PARP-dependent cell death induced by metformin is the result of oxidative damage to mitochondrial DNA, activation of mitochondrial PARP1, and depletion of mitochondrial NAD⁺.
Several interesting questions remain to be answered. For example, why are non-transformed cells and some breast cancer cell lines less sensitive to the cytotoxic effects of metformin? One possibility is that resistant cells are inefficient at taking up and accumulating metformin. This seems unlikely for the resistant breast cancer cell line MDA-MB-231. We previously showed that this line responds to metformin in terms of activation of AMPK and downregulation of cyclin D1 (22). When p27Kip1 is overexpressed this line undergoes cell cycle arrest in response to metformin but is still resistant to the cytotoxic effects of the drug (22). Likewise, metformin slows proliferation of non-transformed MCF10A cells but does not cause cell death. This suggests that resistance to metformin-induced cell death is not due to a lack of uptake and is also independent of effects on cell cycle progression. Since complex I of the mitochondrial electron transport chain is a primary target of metformin, and all the sensitive breast cancer cells accumulate enlarged mitochondria, it is likely that differences between sensitive and resistant cell lines reside within this organelle. Several possibilities exist. Resistant cells may have higher levels or activity of the mitochondrial superoxide dismutase, MnSOD, allowing them to more effectively eliminate complex I derived superoxide and prevent damage to mitochondria. Many breast cancers express lower levels of MnSod than normal surrounding tissue (63). The cell lines MCF10A and MDA-MB-231, both resistant to metformin cytotoxicity, express higher levels of MnSOD than MCF7 and T47D which are both sensitive to metformin (64). Resistant cells may have more efficient mechanisms for preventing or repairing oxidative damage caused by superoxide production within mitochondria, thus limiting initiation of apoptotic and PARP-dependent cell death pathways. Recent findings suggest that mitochondrial
integrity is maintained by removal of damaged mitochondria through autophagy (65-69). This process, called mitophagy, is thought to be coupled to mitochondrial fission and to involve specific recognition of damaged mitochondria by the autophagic machinery. Inefficient removal of damaged mitochondria though mitophagy could contribute to metformin-induced cell death. Another possibility is that sensitive cells have reduced capacity for maintaining cellular or mitochondrial NAD⁺ levels, perhaps related to changes in NAMPT expression. These and other possibilities will need to be examined experimentally.

Diabetics taking metformin have a lower risk of cancer (1-3) and metformin has been found to enhance neoadjuvant therapy for diabetic breast cancer patients (4). Metformin also inhibits growth of breast tumors in preclinical mouse models (7-9). Thus, an interesting question is whether apoptosis, PARP-dependent cell death, or both, play a role in the inhibitory effects of the drug on cancer development and tumor growth. This can be addressed, in mouse xenograft models, using shRNA technology to knockdown key factors in the cell death pathways combined with specific inhibitors of PARP or caspases. Such studies will provide essential information concerning the potential use of metformin and related drugs in the treatment of cancer and how they may be used to complement other therapeutic approaches.

**Acknowledgements:**

We thank Dr. W. Lee Kraus for providing the MCF7 shLuciferase and shPARP1 stable cell lines.
References


Figure Legends

Figure 1. Metformin induces cell death in breast cancer cells. A. MCF7 cells were treated with metformin (8mM) for the indicated time. Live cells were counted using a trypan blue exclusion assay to obtain a growth curve for control (solid line) and metformin treated cells (dashed line). B. MCF7 and T47D were treated without (-) or with 8 mM metformin (+) for 3 days and MDA-MB-453, BT474, MDA-MB-231 and MCF10A were treated for 4 days. The percent of dead cells in the population was determined by trypan blue exclusion.

Figure 2. Cell death caused by metformin in MCF7 cells has features of apoptosis. A. MCF7 cells were treated with (+) or without (-) metformin for 1 (D1), 2 (D2), or 3 (D3) days and then western blotting was performed to detect cleaved PARP1, cleaved caspase 7, and β-actin. B. Left panel: MCF7 cells were treated with (Met) or without (Con) metformin for 3 days and cells were stained with PI and Annexin V-FITC (top panels). Cells were visualized by fluorescence microscopy using a dual-filter set that allows simultaneous detection of both fluorescent signals. Photographs were taken at 100 X magnification. Green indicates Annexin V positive cells and red indicates PI positive cells. Bottom panels show phase contrast images of the same cultures. Right panel: MCF7 cells were treated with metformin (8 mM) for three days and cells were stained with PI and Annexin V-FITC and analyzed by flow cytometry. Percentage of Annexin V positive cells is shown. C. MCF7 cells were treated with (+) or without (-) metformin (8mM) in the presence or absence (DMSO) or a pan-caspase inhibitor (Q-Val-Asp-OPh, 10 μM) for 2.5 days. Western blotting was performed to examine cleaved...
PARP1 and caspase 7. β-Actin was detected as a loading control. D. MCF7 cells were treated as in C for either 2.5 days (left panel) or 4 days (right panel). The percentage of dead cells in each culture was obtained using the trypan blue exclusion assay.

Figure 3. Metformin causes enlargement of mitochondria in breast cancer cells that are sensitive to metformin treatment. A. MCF7 and T47D were treated with (Met) or without (Con) metformin for 3 days and MDA-MB-453, BT474, MDA-MB-231, and MCF10A were treated for 4 days. Photographs are phase contrast images taken at 200X magnification. B. MCF7 cells were treated as in A and cells were fixed and processed for transmission electron microscopy. Photographs were taken at the magnification indicated in each panel. C. MCF7 cells were electroporated with a plasmid encoding DsRed-Mito. Cells were treated with (Met) or without (Con) metformin for 2.5 days. Cells were fixed and observed under confocal microscopy. Both panels were photographed at the same magnification.

Figure 4. Metformin induces PARP-dependent cell death in MCF7 cells. A. Left panel: MCF7 cells were treated for 4 days with (+) or without (-) metformin in the presence of either vehicle (DMSO) or PARP inhibitor (PARP Inh). Right panel: MCF7 cells were treated for 4 days with (+) or without (-) metformin in the presence of a pan-caspase inhibitor (Q-Val-Asp-OPh, 10 μM) and either vehicle (DMSO) or PARP inhibitor (PARP Inh). The percentage of dead cells in each dish was obtained via trypan blue exclusion assay. A t-test was used to determine statistical differences between the indicated groups (* indicates a P value < 0.02; ** indicates a P value < 0.001). B. MCF7
cells stably expressing either a control shRNA (shLuc) or an shRNA targeting PARP1 (shPARP) were treated with metformin and pan-caspase inhibitor as indicated for either 2 days (left panel) or 3 days (right panel). The percentage of dead cells in each dish was obtained via trypan blue exclusion assay. **C.** Extracts from cells treated as in B were analyzed by western blotting for PARP1, cleaved caspase 7, full-length caspase 7, and β-actin.

**Figure 5.** Metformin-induced mitochondrial enlargement is associated with PARP-dependent cell death. **A.** MCF7 cells were treated with or without (Control) metformin for 2.5 days in the presence of vehicle (No Inhibitor), pan-caspase inhibitor, PARP inhibitor, or both pan-caspase inhibitor and PARP inhibitor. Phase contrast photomicrographs were taken at 200X magnification. **B.** MCF7 cells expressing pAcGFP1-Mito were treated as in A and observed by confocal microscopy. **C.** MCF7 cells stably expressing either a control shRNA (shLuc) or an shRNA targeting PARP1 (shPARP) were treated for 2 days with or without metformin in the presence of DMSO (Vehicle) or caspase inhibitor. Phase contrast photomicrographs were taken as in A.

**Figure 6.** Metformin stimulates PAR synthesis in sensitive cells. **A.** MCF7 (left panel) or MDA-MB-231 (right panel) cells were treated with (Met) or without (Con) metformin for 2.5 days and then PAR (red) was detected by immunofluorescence using confocal microscopy. Nuclei (blue) were stained with DAPI. **B.** MCF7 cells were treated with or without metformin in the presence of DMSO or PARP inhibitor for 2.5 days. PAR (red) and nuclei (blue) were detected as described in A.
Figure 7. AIF plays a role in mediating sensitivity to metformin and the drug promotes AIF release from mitochondria and uptake into nuclei. A. MCF7 cells were treated with or without metformin in the presence or absence of PARP inhibitor for 2.5 days. Cells were then fixed and immunofluorescence was used to detect endogenous AIF (green signal). B. MCF7 cells were treated with (Met) or without (Con) metformin for 2.5 days and then nuclear levels of AIF were examined by western blotting. Sp1, a transcription factor, was used as a nuclear marker and loading control. C. MCF7 cells were transfected with an siRNA that targets AIF (siAIF) or a non-targeted control siRNA (Scramble). One day after transfection the cells were treated with (M) or without metformin (C) and treated with vehicle (DMSO) or pan-caspase inhibitor (Casp Inh). After 3 days the cells were harvested and analyzed by western blotting to detect AIF or β-actin. D. The number of live and dead cells in cultures transfected and treated as described in B was determined by trypan blue exclusion assays. A t-test was used to determine statistical differences between the indicated groups (* indicates a P value < 0.05).

Supplementary Figure 1. A. MCF7 cells were treated with either DMSO or the indicated caspase inhibitor (10 μM) in the presence (+) or absence (-) of metformin (Met). After 2 days the cells were harvested and western blotting was used to detect full-length caspase 7 (Casp7), cleaved caspase 7, or β-actin. B. MCF7 cells were treated with vehicle (DMSO), pan-caspase inhibitor (Casp Inh), or PARP inhibitor (PARP Inh) in the presence of absence of metformin. After 1.5 days (left panel), 2.5
days (middle panel), or 3.5 days (right panel) the number of live and dead cells in the cultures was determined using the trypan blue exclusion assay.
Molecular Cancer Research

Metformin Induces Both Caspase-Dependent and Poly(ADP-ribose) Polymerase-Dependent Cell Death in Breast Cancer Cells

Yongxian Zhuang and Keith Miskimins

*Mol Cancer Res* Published OnlineFirst March 21, 2011.

Updated version

Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-10-0343

Supplementary Material

Access the most recent supplemental material at:
http://mcr.aacrjournals.org/content/suppl/2011/05/17/1541-7786.MCR-10-0343.DC1

Author Manuscript

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.