Activation of AMP-activated protein kinase by human EGF receptor 2/EGF receptor tyrosine kinase inhibitor protects cardiac cells

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The human EGF receptor (HER) 2 receptor tyrosine kinase is a survival factor for human cardiomyocytes, and its inhibition may explain the increased incidence of cardiomyopathy associated with the anti-HER2 monoclonal antibody trastuzumab (Genentech, South San Francisco, CA), particularly in patients with prior exposure to cardiotoxic chemotherapies e.g., anthracyclines. Here, we show that GW2974 (HER2/EGF receptor tyrosine kinase inhibitor), but not trastuzumab, activates AMP-activated protein kinase (AMPK), initiating a metabolic stress response in human cardiomyocytes that protects against TNF α -induced cell death. GW2974 stimulates calcium dependent fatty acid oxidation *in vitro* and in the myocardium of GW2974-treated rodents. Calcium chelation or siRNA-targeted AMPK knockdown blocks GW2974 induced fatty acid oxidation. In addition, inhibition of AMPK by a specific inhibitor resulted in increased killing of cardiomyocytes. Elucidating the effects of HER2-targeted therapies on AMPK may predict for risk of cardiomyopathy and provide a novel HER2-targeted strategy designed to protect myocardium from the pro-apoptotic effects of pro-inflammatory cytokines released in response to cardiac injury by chemotherapy or acute ischemia.

monoclonal antibodies

The human EGF receptor (HER) 2 receptor tyrosine kinase plays an essential role in cardiac development during embryogenesis and as a survival factor in adult myocardium (1–4), as evidenced by the increased incidence of cardiomyopathy associated with trastuzumab (Herceptin), a humanized anti-HER2 monoclonal antibody used to treat HER2-overexpressing breast cancers (5, 6). Although inhibition of HER2 causes mitochondrial dysfunction in cardiomyocytes (3, 7, 8), the exact mechanism responsible for trastuzumab-induced cardiomyopathy is unknown. Other HER2-targeted therapies, with different mechanisms of action, do not appear to have the same risk of cardiomyopathy. For example, lapatinib (GW572016) (GlaxoSmithKline, King of Prussia, PA), a small molecule inhibitor of the HER2 and EGF receptor (EGFR) tyrosine kinases, is clinically efficacious in heavily pretreated HER2-overexpressing breast cancers (9), yet it appears to have less risk of cardiomyopathy compared with trastuzumab (10). Interestingly, a non-HER receptor tyrosine kinase inhibitor, imatinib (Novartis, East Hanover, NJ), which targets members of the c-abl family, was recently shown to induce heart toxicity (11–13), raising concerns over the cardiac safety of other small molecule tyrosine kinases.

Under normal physiologic conditions, the adult myocardium utilizes fatty acid or glucose oxidation as its main energy source, with fatty acid oxidation transcriptionally regulated by members of the nuclear receptor superfamily, including the peroxisome proliferator-activated receptors (PPARs) and their coactivators, PPAR γ coactivators-1 α (PGC-1 α) (14–17). Orphan nuclear receptors have also been implicated in regulating cardiac energy metabolism, including the three members of the estrogen-related receptor

(ERR) family ERR α , ERR β , and ERR γ (18). In adults, ERR α and ERR γ expression is enriched in tissues that rely primarily on mitochondrial oxidative metabolism for energy, such as heart, brown adipose, and slow-twitch skeletal muscle. ERR α likely plays a role in lipid catabolism in heart, consistent with its functional interaction with PGC-1 α , making it a critical regulator of energy metabolism (18–24).

Under stress conditions, such as hypoxia, ischemia, glucose deprivation, and starvation, an increase in the intracellular AMP:ATP ratio allosterically activates AMP-activated protein kinase (AMPK), a response designed to maintain cellular energy balance (23, 25). AMPK was initially discovered as an activity that inhibited preparations of acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase. Activation of AMPK initiates a series of downstream phosphorylation events that switch cells from active ATP consumption (e.g., fatty acid, cholesterol, and protein biosynthesis) to ATP production (e.g., fatty acid and glucose oxidation) (26, 27). Stress-induced activation of AMPK occurs after its phosphorylation at threonine 172 on the α subunit by one or more upstream AMPK kinases, including calmodulin-dependent kinase kinase β , a calcium-activated protein kinase (28), and LKB1, a serine/threonine kinase encoded by the Peutz-Jegher's syndrome tumor suppressor gene (29–32). Activation of AMPK in skeletal muscle and heart leads to the phosphorylation and inhibition of ACC, which in turn reduces the level of malonyl-CoA, itself an inhibitor of carnitine palmitoyltransferase I (CPT I). Derepression of CPT I results in the concomitant increase in β -oxidation of fatty acid, leading to increased mitochondrial production of ATP (26, 27, 33). Stress-induced activation of AMPK also inhibits protein synthesis by inhibition of mTOR and directly modulating elongation factor (eEF)2, a translation elongation factor known to be associated with cardiac protection (31, 34–36). Importantly, alteration in mitochondrial function has been reported to lead to cardiomyocyte death by imatinib (11). Moreover, inhibition of cap-dependent translation by AMPK-mediated TSC2 phosphorylation appears to be critical for cell survival in response to ATP depletion (32). Increased biosynthesis of (rather than consump-

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Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; eEF, elongation factor; EGFR, epidermal growth factor receptor; ERR, estrogen-related receptor; HER, human epidermal growth factor receptor; HMCs, human myocardiocytes; PGC, peroxisome proliferator-activated receptors coactivator.

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Fig. 1. GW2974 stimulates increased intracellular calcium levels in GW2974-treated HMCs and Au565. HMCs and Au565 were preloaded with Fluro-4 dye and then treated with 25 μ M GW2974. Images were then taken by confocal microscopy after treatment with GW2974. Cells treated with vehicle alone served as controls and baseline autofluorescence was assessed in untreated cells. These results are representative of three independent experiments.

tion of) ATP following AMPK activation may also protect cardiomyocytes against ischemic injury (36).

Here, we demonstrate that GW2974, a potent small molecule HER2/EGFR tyrosine kinase inhibitor with a similar profile to lapatinib (37) [supporting information (SI) Table 1], activates AMPK and its downstream substrates, and stimulates fatty acid oxidation, which in turn increases ATP production in HER2-expressing human cardiomyocytes, protecting against apoptosis induced by TNF α , a known cytokine detected in cardiac failure (38). Conversely, trastuzumab does not activate AMPK. Instead, it leads to enhanced cardiomyocyte cell death in response to TNF α . The effects of specific HER2-targeted therapies on AMPK and consequently energy production may predict for the risk associated cardiomyopathy and provide a novel HER2-directed therapeutic strategy to protect myocardium from the killing effects of TNF α or other proapoptotic stimuli, following acute ischemic injury.

Results

GW2974 Modulates the Activity of Genes Involved in Regulating Fatty Acid Metabolism and Calcium Channels in HER2-Expressing Cancer Cells and Human Myocardocytes (HMCs). To better understand the cardiac sparing effects of HER2 kinase inhibitors like lapatinib and GW2974, we assessed the effects of GW2974 on gene expression in the HER2-overexpressing Au565 breast cancer cell line, a cell line sensitive to HER2 kinase inhibition. We found that GW2974 modulated the activity of a number of genes associated with calcium/ion channels and fatty acid metabolism (SI Tables 2–4). The increased activity of genes involved in calcium-mediated activities and ion channels led us to investigate the effects of GW2974 on intracellular calcium levels in HER2 expressing cells. HMCs, which express physiological levels of HER2 and Au565 cells, were preloaded with Fluro-4 dye, treated with GW2974, and then scanned by using fluorescent microscopy. Intracellular calcium increased rapidly in GW2974-treated cells (Fig. 1).

GW2974 is an equipotent inhibitor of the HER2 and EGFR tyrosine kinases similar to lapatinib (37) (SI Table 1). To determine whether the effect of GW2974 on calcium flux was HER2 or EGFR-dependent, we compared intracellular calcium levels in parental MCF7 breast cancer cells, which express EGFR and only low levels of HER2 and are resistant to the anti-tumor effects of HER2 kinase inhibitors (39). We also studied MCF7/HER2 cells, a stably transfected MCF7 line that overexpresses HER2. In contrast to parental MCF7 cells where intracellular calcium levels essentially remained unchanged, treatment with GW2974 resulted

Fig. 2. GW2974 stimulates calcium-dependent activation of AMPK and its downstream substrates that regulate fatty acid oxidation and protein synthesis in response to HER2 kinase inhibition by GW2974. (a) HER2-positive HMCs and the HER2-overexpressing Au565 breast cancer cell line were treated with GW2974 (25 μ M) or trastuzumab (50 μ g/ml) for 15 min. Equal amounts of protein from total cell lysate were separated by SDS/PAGE, and steady-state protein levels were assessed by Western blot as indicated. Steady-state protein levels of phospho-AMPK α and its downstream substrates, ACC and eEF2, increased in GW2974 but not trastuzumab-treated HMCs and Au565 cells. Activation of AMPK α in HMCs occurred despite steady-state phosphorylated Akt (p-Akt) protein levels remaining unchanged. Actin steady-state protein levels served as a control for equal loading of protein. These results are representative of three independent experiments. The HMCs or Au565 cells were treated with or without GW2974 and lapatinib (25 μ M or 50 μ M) and were tested. (b) HMCs cells were treated with the intracellular calcium chelator BAPTA/AM (30 μ M) for 30 min before adding GW2974 (25 μ M) for an additional 15 min. Phospho-AMPK α steady protein levels were then determined in cells treated with vehicle alone (lane 1), GW2974 (lane 2), BAPTA/AM alone (lane 3), or BAPTA/AM and the GW2974 (lane 4). Pretreatment with BAPTA/AM blocked the induction of phosphorylated AMPK α (p-AMPK α) protein in GW2974-treated cells. Results are representative of three independent experiments.

in a 5- to 8-fold increase in intracellular calcium in MCF7/HER2 cells (SI Fig. 7). Increased intracellular calcium levels in response to GW2974 appear to require HER2 expression, although a supportive role of EGFR or HER3 as heterodimeric partners to HER2 cannot be excluded.

GW2974, but Not Trastuzumab, Activates AMPK and Its Downstream Substrates in Breast Cancer Cells and HMCs. GW2974 affected the activity of genes involved in cellular metabolism, calcium/ion channels, and calcium/calmodulin-dependent proteins (SI Tables 2–4). Furthermore, calmodulin-dependent kinase kinase β , a calcium-regulated serine/threonine kinase, activates AMPK, a master regulator of cell metabolism and energy production that is induced by a variety of stress stimuli, including an increase in the AMP:ATP ratio. We therefore postulated that GW2974 activates AMPK in a calcium-dependent manner and that AMPK activation protects HER2 expressing HMCs from apoptotic stimuli.

To test this hypothesis, we compared the activation state of AMPK and its downstream substrates in HMCs and Au565 cells treated with either GW2974 or trastuzumab. Importantly, GW2974, but not trastuzumab, increased steady-state protein levels of activated, phosphorylated AMPK in HMCs and Au565 cells (Fig. 2a). As a consequence of AMPK activation, eEF2 and ACC were phosphorylated in GW2974 but not in trastuzumab-treated cells. Inhibition of PI3K-Akt signaling is a potent activator of AMPK. However, AMPK was activated in GW2974-treated HMCs despite very little change in the steady-state protein levels of phosphorylated Akt (Fig. 2a) indicating that AMPK activation in response to GW2974 is Akt-independent. The lack of inhibition of phosphorylated Akt in GW2974-treated HMCs compared with Au565 cells is consistent with the dependence of Akt activation on HER2 signaling in the HER2-overexpressing Au565 cells compared with HMCs, which express physiological levels of HER2 and where Akt activation is likely maintained by redundant survival pathways. In addition, we were able to show that survival of HMCs depends

Fig. 3. Activation of AMPK by GW2974 in turn activates mediators of fatty acid oxidation, resulting in reduced cellular lipid content, which is calcium-dependent. (a) HMCs cells were subjected to the indicated treatment conditions (see Fig. 2a) for 3 days, and then cellular lipid content was determined by Oil Red-O staining and light phase microscopy (100X). GW2974, but not trastuzumab, reduced cellular lipid content, which was blocked by pretreatment with BAPTA/AM. These results are representative of three independent experiments. (b) Selective AMPK knockdown blocked the effects of GW2974 on cellular lipid content compared with scrambled siRNA controls. Cells were untreated or treated with GW2974 together with either AMPK siRNA or scrambled siRNA and stained with Oil Red-O to assess cellular lipid content after two doses. (c) Steady-state protein levels of ERR α , PGC-1, and MCAD were increased in GW2974-treated cells; HMCs were treated for 2 days as described in Fig. 2a, and then steady-state protein levels of the indicated proteins were assessed by Western blot. GW2974, but not trastuzumab, increased steady-state protein levels of PGC-1 and ERR α , molecules associated with mitochondrial fatty acid oxidation and MCAD, an inhibitor of fatty acid synthesis. These results are representative of three independent experiments.

AMPK activation as treatment with compound C, a specific inhibitor of AMPK, induced cell death even in the presence of GW2974 (SI Fig. 8). The activation of AMPK depended on HER2 in Au565 but not in HMCs cells (SI Fig. 9a and b), because the addition of heregulin abolished AMPK activation only in Au565 cells.

To further define the calcium-dependent activation of AMPK in response to GW2974, HMCs and HER2-overexpressing breast cancer cells were pretreated with BAPTA/AM, a calcium chelator. Treatment with BAPTA/AM alone did not activate AMPK. However, pretreatment with BAPTA/AM completely blocked the activation of AMPK by GW2974 (Fig. 2b). These findings indicate that inhibition of HER2 kinase by GW2974 is calcium-dependent.

Activation of Fatty Acid Oxidation and Change in Lipid Content in GW2974-Treated HMCs Is Calcium and AMPK-Dependent. Activation of AMPK stimulates increased fatty acid oxidation and ATP production. We compared the oxidation of fatty acids in HMCs treated with GW2974 or vehicle alone. GW2974 markedly reduced acylcarnitine C18 and C16 with a consequential increase in smaller lipid oxidation products (C2) (SI Table 5). We next determined the effects of GW2974 on cellular lipid content in HMCs. Using Oil Red-O staining to assess cellular lipid content, we found that lipid content was markedly reduced in GW2974 (but not trastuzumab) treated HMCs (Fig. 3a). Increased fatty acid oxidation and reduction in cellular lipid content in response to GW2974 was calcium-dependent as these biological effects were blocked by pretreatment with BAPTA/AM (Fig. 3a).

Although these results implicate the activation of AMPK in the induction of fatty acid oxidation and reduction in cellular lipid content in GW2974-treated cells, they do not demonstrate a direct cause and effect relationship. To directly demonstrate the functional role of AMPK in fatty acid oxidation and changes in lipid content in GW2974-treated HER2 expressing cells, we selectively knocked down AMPK, using siRNA transfection. Steady-state AMPK protein levels were effectively reduced by AMPK siRNA (SI Fig. 10). AMPK knockdown blocked the induction of fatty acid oxidation in response to GW2974 compared with cells transfected with a control (scrambled) siRNA construct (Fig. 3b).

In addition to the changes in lipid content, treatment with GW2974 increased steady-state protein levels of PGC-1 and ERR α , molecules associated with mitochondrial fatty acid oxidation, and MCAD, an inhibitor of fatty acid synthesis (Fig. 3c), whereas trastuzumab failed to do so. Thus, GW2974 triggers a cascade of metabolic events mediated by calcium-dependent activation of AMPK, resulting in downstream events that conserve cellular

energy by inhibiting fatty acid synthesis while activating fatty acid oxidation as an alternative source of energy.

GW2974 Induces Fatty Acid Oxidation and Changes in Lipid Content and Protect Cells from Apoptosis in the Myocardium of GW2974-Treated Rats. We next determined whether the effects of GW2974 on cellular lipid content in HMCs also occur *in vivo*. Eight-week-old Sprague–Dawley rats were first starved for 12 h and then administered a single dose of GW2974 (100 mg/kg) or vehicle by oral gavage. Eight hours after treatment, the animals were killed, their hearts were harvested by snap freezing, and then myocardial lipid content was assessed by Oil Red-O staining.

Lipid staining was markedly reduced in the myocardium of GW2974-treated rats compared with those administered vehicle alone (Fig. 4a). Because AMPK is also activated in response to nutrient deprivation (starvation) and can affect mitochondrial TCA based metabolism, we tested the apoptotic activity in the myocardium of starved rats administered GW2974 or vehicle alone.

Fig. 4. Myocardial lipid content is reduced in GW2974-treated rats, which is associated with protection against metabolic stress-induced apoptosis. (a) Eight-week-old female Sprague–Dawley rats were treated as described in Methods. Briefly, rats were fasted for 12 h before treatment with 100 mg/kg of GW2974 for 8 h or left untreated and then were killed. Their hearts were harvested and snap frozen, and sections were stained for lipid by Oil Red-O and examined by light microscopy ($\times 40$). Notice the abundant lipid in untreated rats compared with those treated with GW2974. (b) Frozen sections of hearts from untreated or GW2974-treated rats were stained by hematoxylin and eosin and for apoptosis by TUNEL staining. Notice the brown stained apoptotic nuclei (TUNEL positive marked with an arrow) in the untreated rats (5%) compared with the absence of significant apoptosis ($<1\%$) in the myocardium of rats treated with GW2974.

GW2974, but Not Trastuzumab, Increases ATP Production and Protects Against TNF α -Induced HMCs Cell Death. Pro-inflammatory cytokines (e.g., TNF α) are frequently elevated in cancer patients, a potential contributing factor to the cardiotoxic effects of trastuzumab. In this regard, we speculated that AMPK activation and stimulation of lipid oxidation in GW2974-treated HMCs might protect against TNF α induced cell death.

Activation of AMPK reduces energy consumption while stimulating alternate metabolic pathways that lead to increased ATP production. There were marked differences in cellular ATP levels in GW2974-treated HMCs compared with cells treated with trastuzumab or TNF α . In three independent experiments with a total of 11 data points, ATP levels were significantly increased in GW2974-treated HMCs ($P = 0.002$) compared with reduced ATP levels in trastuzumab ($P = 0.007$) and TNF α treated cells ($P < 0.0001$) (Fig. 5b). The ability to activate fatty acid oxidation and consequently maintain or increase intracellular ATP content may be a critical factor mediating the cardioprotective effects of short-term exposure to GW2974.

In addition to their disparate effects on AMPK, GW2974 and trastuzumab differ in their ability to activate the anti-apoptotic NF κ B survival pathway in HMCs. TNF α is a potent activator of NF κ B/RelA, as evidenced by the increased steady-state protein levels of phospho-NF κ B in HMCs (SI Fig. 11). Concomitant treatment with GW2974 did not affect the activation of NF κ B by TNF α . However, steady-state phospho-NF κ B protein levels were markedly reduced in cells treated concomitantly with TNF α and trastuzumab. The inhibition of protein synthesis by phosphorylation of eEF2 and the activation of the prosurvival factor NF κ B as well

One possibility is that small molecule kinase inhibitors like GW2974 and lapatinib have more potent inhibitory effects on HER2 signaling, with a more pronounced effect on HER2 transactivation of HER3 (41). HER2:HER3 heterodimers are the most potent activators of survival pathways, because HER3 has six binding sites for subunits of PI3K (42, 43). Trastuzumab was recently shown to inhibit signaling via EGFR:HER2 heterodimers but not HER2:HER3 complexes (44). The increased intracellular calcium levels resulting in subsequent activation of AMPK and fatty acid oxidation in GW2974-treated cells, but not in trastuzumab-treated cells, may reflect the more potent effects of a small molecule HER2 tyrosine kinase inhibitor on HER2:HER3 complex activation compared with an antibody-based therapy like trastuzumab (Fig. 6). Preliminary results in our laboratory favor this hypothesis. The involvement of protein tyrosine kinases in ion and calcium channels are reported in ref. 45.

stock solution was prepared by dissolving 300 mg of the dye in 100 ml of 2-propanol by heating to 100°C with stirring. Working solution was prepared by combining six parts of stock solution with four parts of water.

Apoptosis Analysis. Apoptosis staining was performed by the TUNEL staining procedure as described in ref. 9.

Calcium Detection. Au565, MCF7, MCF7/HER2, and HMCs cells were seeded on glass bottom dishes (MatTek, Ashland, MA) the day before the experiment. On the day of experiment, a 1:1 mixture of Ca^{2+} indicator Fluo-4 (Molecular Probes, Carlsbad, CA) (5 μM) and Pluronic was diluted with regular growth medium and used to replace the original media. Incubation with Fluo-4 was 30 min at 37°C. Afterward, cells were washed in 37°C PBS and incubated in Phenol Red-free medium for 15 min. Then 2 $\times$ solution of the drug in Phenol Red-free medium was made and mixed gently near the microscope (Yokogawa Nipkow spinning disk confocal built on a Nikon TE-2000U microscope at Northwestern University, Chicago, IL).

ATP Measurements. ATP determination was done by using an ATP Bioluminescence Assay Kit HS 2 (Roche, Basel, Switzerland). Briefly, human cardiomyocytes were grown and treated with the drugs on six-well culture dishes. On the day of the experiment, cells were trypsinized, spun down, and resuspended in the dilution buffer. Equal amounts of the cell suspension and lysis buffer were mixed gently in Eppendorf (Boulder, CO) tubes and incubated for 5 min, and aliquots were transferred into 96-well microplates (white, nontransparent) with luciferase reagent. ATP was measured immediately by using a Dynatech (Chantilly, VA) ML 1000 Plate Reader. The ATP standard curve was prepared by using ATP provided with the kit.

siRNA Transfection. siRNA to AMPK was purchased from Dharmacon (Lafayette, CO). siRNA duplex for scrambled control

siRNA was synthesized (IDT, Coralville, IA) as follows: sense 5' rGrCrGrCrGrCrUrUrUrGrUrArGrGrArUrUrCrGTT and anti-sense 5' rCrGrArArUrCrCrUrArCrArArArGrCrGrCrGrCTT. The duplex was resuspended according to the manufacturer's recommended protocol. Primary HMCs were transfected with 2 ng of AMPK or scrambled siRNA, using Nucleofector (Amata, Gaithersburg, MD) and the Rat Cardiomyocyte Neo Nucleofactor Kit. After transfection the cells were seeded into (poly)L-lysine coated chamber slides. The following day, the cells were either left untreated or treated with 25 μM GW2974 for 3 days and then stained for lipids.

In Vivo Studies. Female Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN). At 8 weeks, the rats were treated. Before treatment, the rats were put under fast for 12 h, administered GW2974 100 mg/kg by oral gavage, and killed 8 h later, at which time their hearts were harvested and snap frozen. GW2974 was obtained from Sigma and dissolved in 0.5% hydroxypropylmethylcellulose/0.1% Tween 80/99.6% water for dosing. To prepare the dosing solution, the required amount of vehicle or GW2974 was added and mixed thoroughly. The final dosing suspension was a bright yellow fine suspension with pH 4.8. The rats were housed in Thoren (Hazleton, PA) microisolator caging with Bed-O-Cobs bedding. All procedures carried out in this experiment were approved by the MIR Animal Care and Use Committee.

Statistical Analysis of ATP Levels. Analysis was performed by using SPSS software version 13.0 (SPSS, Chicago, IL), using Fisher's exact test for ATP levels. We assumed the control value to be 100% in all cases. The percent difference from the control was calculated for each data-point and the statistical significance of the difference between the treatment and the control was assessed.

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